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Oklahoma State Univ., Stillwater

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BIOCHEMICAL ANALYSIS FOR DETECTION AND ASSESSMENT OF POLLUTION IN THE SUBSURFACE ENVIRONMENT

by

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16. ABSTRACT

Selected biochemical analysis techniques were investigated for potential use in detecting and assessing pollution of subsurface environments. Procedures for determining protein, nucleic acids, organic phosphate, lipopolysaccharides, and various coenzymes and enzyme systems were evaluated. These procedures were modified and adapted for application to environmental samples, and sensitivities were determined in terms of numbers of Escherichia coli cells which could be detected.

Standard spectrophotometric and fluorimetric methods for protein, DNA, RNA, and organic phosphates lacked sufficient sensitivity for successful application to subsurface environmental samples. Methods for coenzymes and enzymes which employed enzymatic cycling procedures could be made highly sensitive, but required use of very sophisticated and difficult micro-procedures. The Limulus amebocyte lysate test, which embodies a built-in amplification since lipopolysaccharide activates an enzyme which then catalyzes the reaction to be measured, and the bioluminescence or chemiluminescence procedures (firefly luciferase, bacterial luciferase (Microtox), luminol), which provide an order of magnitude increase in sensitivity over fluorimetric procedures, were highly promising. These methods are currently applicable to many environmental samples, and it should be possible to significantly increase their sensitivity, reliability, and applicability by further study.

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Although the research described in this report has been funded wholly or in part by the United States Environmental Protection Agency through grant number R-804613 to Oklahoma State University - Oklahoma Agricultural Experiment Station, it has not been subjected to the Agency's peer and policy review and therefore does not necessarily reflect the views of the Agency and no official endorsement should be inferred. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

FOREWORD

EPA is charged by Congress to protect the Nation's land, air and water systems. Under a mandate of national environmental laws focused on air and water quality, solid waste management and the control of toxic substances, pesticides, noise, and radiation, the Agency strives to formulate and implement actions which lead to a compatible balance between human activities and the ability of natural systems to support and nurture life. In partial response to these mandates, the Robert S. Kerr Environmental Research Laboratory, Ada, Oklahoma, is charged with the mission to manage research programs to investigate the nature, transport, fate, and management of pollutants in ground water, to develop and demonstrate technologies for treating wastewaters with soils and other natural systems and for controlling pollution from irrigated crop and animal production agricultural activities, and to develop and demonstrate cost-effective land treatment systems for the environmentally safe disposal of solid and hazardous wastes.

This report describes the evaluation of selected analytical biochemical methods for applicability in detecting the presence and determining the potential for biodegradation of pollutants in unsaturated soil profiles and ground waters. Detailed analytical protocols are presented for a number of procedures which have been optimized and evaluated in terms of complexity, dynamic range, and sensitivity. Several of these procedures exhibit particular promise for detection and quantitation of subsurface biological activity and should be of significant value in the development of reliable predictive methodologies needed for the control and alleviation of ground water pollution. They may also find application in investigations of biological contamination of ground water and in assessment of the impact of pollutants on subsurface biological communities. In addition, the compilation of optimized analytical biochemical techniques and literature references presented should be generally useful for investigations requiring determination of biological parameters in environmental media, such as those involving detection and evaluation of ecosystem perturbations resulting from environmental pollution.

Clinton W. Hall

Clinton W. Hall, Director
Robert S. Kerr Environmental
Research Laboratory

PREFACE

The goal of the research described in this report was to establish which of many analytical biochemistry procedures could be applied for detecting and assessing subsurface pollution.

First, we established our capability for obtaining reproducible results using the published procedures. Any modifications that we found necessary and a detailed protocol for each test is presented in the Experimental Procedures Section. This section should be of value to all researchers who want a compilation of analytical techniques.

In the Results and Discussion Section we present standard curves and establish the limit of detection possible in terms of numbers of Escherichia coli cells. We discuss the major advantages and disadvanatges of the methods and present a comparison. Any new methods or useful modifications that have appeared recently are cited.

This experimental exposition is a companion to our theoretical discussion in Biochemical Indicators of Subsurface Pollution by Otis C. Dermer, Vivian S. Curtis, and Franklin R. Leach and published by Ann Arbor Science Publishers, 1980 (Library of Congress Catalog Card No. 80-67658 and ISBN 0-250-40383-8).

ABSTRACT

Selected biochemical analysis techniques were investigated for potential use in detecting and assessing pollution of subsurface environments. Procedures for determining protein, nucleic acids, organic phosphate, lipopolysaccharides, and various coenzymes, enzymes, and enzyme systems were evaluated. These procedures were modified and adapted for application to environmental samples, and sensitivities were determined in terms of the number of Escherichia coli cells which could be detected.

Standard spectrophotometric and fluorimetric methods for protein, DNA, RNA, and organic phosphates lacked sufficient sensitivity for successful application to subsurface environmental samples. Methods for coenzymes and enzymes which employed enzymatic cycling procedures could be made highly sensitive, but required use of very sophisticated and difficult microprocedures. The Limulus amebocyte lysate test embodies a built-in amplification since lipopolysaccharide activates an enzyme which then catalyzes the reaction to be measured. The bioluminescence or chemiluminescence procedures <firefly luciferase, bacterial luciferase (Microtox), and luminol> provide an order of magnitude increase in sensitivity over fluorimetric procedures. Both are highly promising. These methods are currently applicable to many environmental samples; further study should make possible a significant increase in their sensitivity, reliability, and applicability.

This report was submitted in fulfillment of Grant # R-804613 by Oklahoma State University under the sponsorship of the U. S. Environmental Protection Agency. This report covers the period August 15, 1976 to March 15, 1981, and work was completed as of November 5, 1981.

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ABBREVIATIONS, UNITS AND SYMBOLS

ABBREVIATIONS

ADP — adenosine diphosphate
ATP — adenosine triphosphate
Bicine — (N,N-Bis(2-hydroxyethyl)glycine)
BSA — bovine serum albumin
DAPI — 4',6-diamidino-1-phenylindole-2 HCl
DBA — 3,5-diaminobenzoic acid
DDT — 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane
DMSO — dimethyl sulfoxide
DNA — deoxyribonucleic acid
DTT — dithiothreitol
EDTA — ethylenediaminetetraacetic acid, Versene
FAD — flavin adenine dinucleotide
FMN — flavin adenine mononucleotide
$FMNH_2$ — reduced flavin mononucleotide
GDH — glutamic acid dehydrogenase
G6PDH — glucose 6-phosphate dehydrogenase
HEPES — 4-(2-hydroxyethylpiperazine-ethanesulfonic acid)
HEPPS — 4-(2-hydroxyethyl)-1-piperazine-propane sulfonic acid
INT — 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride
INTF — reduced 2-iodophenyl-3-nitrophenyl-5-phenyltetrazolium chloride (formazan)
LAL — *Limulus* amebocyte lysate
LDH — lactic acid dehydrogenase
LPS — lipopolysaccharide
MOPS — 4-(N-morpholine)propanesulfonic acid
NAD^+ — nicotinamide adenine dinucleotide (oxidized)
$NADH_+$ — nicotinamide adenine dinucleotide (reduced)
$NADP^+$ — nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH — nicotinamide adenine dinucleotide phosphate (reduced)
OSU — Oklahoma State University
PEP — phosphoenolpyruvic acid
P_i — Inorganic phosphate
RNA — ribonucleic acid
TCA — trichloroacetic acid
TES — 2-((Tris(hydroxymethyl)methyl)amino)ethanesulfonic acid
Tricine — (N-Tris(hydroxymethyl)-glycine)
Tris — Tris(hydroxymethyl)aminomethane
TTC — triphenyltetrazolium chloride

UNITS

c -- centi 10^{-2}
m -- milli 10^{-3}
μ -- micro 10^{-6}
n -- nano 10^{-9}
p -- pico 10^{-12}
f -- femto 10^{-15}
M -- molar (moles per liter)
g -- gram
k -- kilo 10^{3}
L -- liter
N -- normal
PPB -- parts per billion

SYMBOLS

A -- absorbance
Δ -- change
V -- velocity

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SECTION 1

INTRODUCTION

Basic to advancement in the understanding of any scientific subject are sensitive, accurate, and facile analytical techniques. The scientific publications that become citation classics are often concerned with the description of an analytical technique or means of presenting data, e.g., the Lowry protein determination and the Lineweaver-Burk plot.

Progress in biochemical research and molecular biology during the last two decades was built on three to four decades of extensive methods development. This time span has progressed from the notion that DNA might be the repository of genetic information to the determination of base sequence and chemical synthesis of genes. While these technical developments are rapidly communicated and applied in basic research, there has been a lag in their application to more applied areas.

Dermer, Curtis, and Leach (1) reviewed potential biochemical indicators of subsurface pollution in soil and ground water. Their book is a general survey of the strategy of applying biochemical analysis to subsurface pollution problems. The promising methods identified in that study have now been tested in our laboratory for application to environmental samples.

This report presents the results of a validation of the procedures in this laboratory, a comparison of the sensitivities of the various methods, and their application to environmental samples. The details of the reagents and methods used are presented herein to serve as a single compilation of the methods. The comparative data and discussion will allow choice of a method to be made more rationally.

SECTION 2

CONCLUSIONS

Current analytical biochemistry procedures are widely applicable and reliable. Colorimetric and spectrophotometric determinations are in routine laboratory use throughout the world. They are of sufficient sensitivity to satisfy most experimental situations, but they are, unfortunately, not sufficiently so to be useful with many sparsely populated environmental samples. Additional sensitivity can be obtained using fluorimetric and/or enzymatic cycling assays, which are within the capabilities of trained biochemists in well-equipped research laboratories. But such sophistication of techniques and elegance of equipment is beyond the personnel, laboratories, and budgets of those obligated to determine the quality of most water samples or subsurface materials. Development of these techniques must be accomplished for the research laboratories and then simplifications, if possible, made to allow use in the quality control laboratories. Bioluminescence and chemiluminescence assays were found to have superior sensitivity and to entail simple instrumentation and procedures.

Determination of ATP using firefly luciferase, analysis for iron porphyrin using the luminol reaction, the Microtox assay based on bacterial luciferase, and the Limulus amebocyte lysate assay were all demonstrated to be useful in environmental sample analysis. Procedures for routine application to many situations were developed and certain assays were optimized. These results are presented.

SECTION 3

RECOMMENDATIONS

Further study on bioluminescent, chemiluminescent, the Limulus amebocyte lysate, and enzymatic cycling reactions will be required to optimize application of these procedures to heterogeneous environmental samples. Specific objectives for additional study on firefly luciferase and Limulus amebocyte lysate assay are listed below. Similar objectives can be formulated for the other assays.

To complete optimization and validation of the firefly luciferase assay for ATP in environmental samples the specific objectives should include:

1. Defining conditions that yield optimum ATP determinations using commercial firefly luciferase preparations.
2. Determining a preferable method for extracting ATP from the microorganisms present in environmental samples of water, soil, and subsurface material.
3. Developing purification procedures that remove luciferase inhibitors present in the extracts and/or developing a means of minimizing their effect and correcting for inhibition.
4. Determining which of the various commercial formulations of firefly luciferase reagents is best with environmental samples.
5. Applying and correlating ATP levels with other indicators of pollution and/or biological activity in environmental samples.

To develop procedures for use of the Limulus amebocyte lysate assay for lipopolysaccharide with application to environmental samples the specific objectives should include:

1. Developing assays with two levels of sensitivity.
   a. A range-finding assay that has a linear response over a wide range of concentrations, which will suggest appropriate dilutions for the more sensitive assay.

b. A more sensitive assay that will detect lipopolysaccharides derived from a minimal number of gram-negative bacteria.

2. Developing an extraction method for the release of the lipopolysaccharides from bacterial cells in environmental samples of soil and subsurface material.
3. Determining the stability and recovery of lipopolysaccharides from environmental samples.
4. Determining whether the extraction procedures also extract materials that are inhibitory to the assay and, if so, developing procedures for minimizing or circumventing any inhibition.
5. Applying and correlating the Limulus amebocyte lysate test with other indicators of pollution and/or biological activity in environmental samples.

SECTION 4

MATERIALS, EQUIPMENT, AND SUPPLIES

The selection, preparation, and characterization of reagents and materials is of critical importance in research and is especially significant with the concentration ranges described in this report. We have listed sources and reagents which we found suitable, but the investigator should assure himself of the quality of his particular lot and of reagents from other sources.

As an example of the care required, we found the following treatment necessary to provide water of sufficient quality for reagent stability and with low background values. The laboratory supplied distilled water, which was obtained by reverse osmosis, was passed through two mixed-bed ion exchange columns, glass distilled from a sterile Bellco still, collected in sterile vessels, filtered through a 0.22 μm membrane filter, and finally autoclaved.

Each lot of enzyme was characterized before use. This characterization involved assays to establish linearity or saturation, low background, absence of interfering substances, and properties consistent with those obtained with previously used lots.

A. Major Equipment

1. Centrifuges
   - Beckman Microfuge B
   - Sorvall GLC-1
   - Sorvall RC-2
   - Sorvall SS-1
2. Dilutors-Pipettors
   - Brinkmann Sample Reagent Dispenser 5211 Dilutor 5232
   - Gilson Pipetman P-20,P-200,P-1000
3. Homogenizer
   - Brinkmann Polytron
4. Photometers
   - Packard Pico-Lite Model 6100 with a Haake G constant temperature bath and circulator
   - JRB Model 3000 with Kinetic Kit, Houston Instrument Omniscribe recorder, and modified sample chamber
5. Spectrophotometers
   - Gilford Model 2000 System with digital readout Model 410 printer Model 4009, and offset Model 208
   - Zeiss PM 6 with printer, recorder Model 355 and programmer PM6
6. Spectrofluorimeter
   - Aminco SPF 125 with Houston Instrument recorder, integrator-timer J4-7462 A, Chem-glo reaction chamber J4-7442A, printer J4-7483, and Tektronix 5000 series oscilloscope

B. Materials

SUBSTANCE	SOURCE
ADP	Sigma
ATP	Sigma
Adenylate kinase	Sigma
Alamine	General Mills
Alkaline phosphatase	Sigma
Bovine serum albumin	Armour
Bromosulfalein	Aldrich
Buffers	
Bicine	Sigma
Borate	Fisher
Glycylglycine	Sigma
HEPES	Sigma, Calbiochem
HEPPS	Sigma
MOPS	Sigma
TES	Sigma

Tricine	Sigma
Tris	Coleman, Matheson & Bell
Imidazole	Sigma
Catalase	Sigma
Citric acid	Mallincrodt
Coomassie Brilliant Blue G-250	Sigma
DNA	Sigma
DBA	Aldrich & Sigma
DAPI	Serva
Diphenylamine	Aldrich
Diaphorase	Sigma
Disodium p-nitrophenyl phosphate	Sigma
DTT	Calbiochem
Ethidium bromide	Aldrich
EDTA	Fisher
Firefly luciferase	ALL
	Boehringer-Mannheim
	Calbiochem
	DuPont
	LKB
	Lumac
	SAI
	Sigma
Folin reagent	Fisher
Freon	Union Carbide
FMN	Sigma
Glucose	Sigma
Glucose-6-phosphate dehydrogenase	Sigma
Glutamate dehydrogenase	Sigma
Hemoglobin	Sigma
Hexokinase	Sigma
Hydrogen peroxide	Fisher
INT	Fisher
INTF	Sigma
LAL	Associates of Cape Cod
LPS	Associates of Cape Cod
Lactate dehydrogenase	Sigma
Lactic acid	Fisher
Luciferin	Calbiochem, Boehringer-Mannheim
2-Mercaptoethanol	Sigma
Microtox reagent	Beckman
NAD^+	Sigma
$NADH_+$	Boehringer-Mannheim
$NADP^+$	Sigma
Orcinol	Aldrich
6-phosphogluconate	Sigma

6-phosphogluconate dehydrogenase	Sigma
o-Phthalaldehyde	Sigma
Pronase	Calbiochem
Pyruvic acid	Sigma
Ribonuclease	Sigma
RNA	Sigma
Sodium deoxycholate	Mann

Other acids, bases, salts, and solvents were reagent grade.

C. SUPPLIERS

Aldrich Chemical Co.
940 W. St. Paul Ave.
Milwaukee, WI 53233

American Instrument Co.
Silver Spring, MD 20910

Analytical Luminescence Laboratories
11180 Roselle St.
Units D & E
La Jolla, CA 92121

Armour
Chicago, IL

Associates of Cape Cod, Inc.
Box 224
Woods Hole, MA 02543

Beckman Instruments Inc.
1117 California Ave.
Palo Alto, CA 94304

Boehringer-Mannheim Biochemicals
Box 50816
Indianapolis, IN 46250

Brinkmann Instruments, Inc.
115 Cutter Mill Rd.
Great Neck, NY

Calbiochem-Behring Corp.
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Houston Instruments
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Austin, TX

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Mallincrodt Chemical Works
St. Louis, MO

Mann Research Laboratories, Inc.
New York, NY

Matheson, Coleman & Bell
Norwood, OH 45512

Packard Instrument Co., Inc.
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Rainin (Gilson) Inst.
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Serva Feinbiochemica

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Biomedical Products
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Fisher Scientific Co.
Fairlawn, NJ

General Mills Chemicals, Inc.
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Gilford Instrument
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New York, NY

SECTION 5

EXPERIMENTAL PROCEDURE

Detailed instructions are given on the reagent preparation and the procedures. The aim is for these instructions to be so complete as to allow unquestioned replication of the experiments. Each reagent is referred to as Reagent <X> to aid in their designation and to simplify what must be written. Each step in a procedure is given a #) to designate it. This allows easier visualization of the complexity of a procedure.

A. Protein Determinations

1. Lowry Protein Determination (2)

Reagents

<A> 2% Na_2CO_3 in 0.1 N NaOH

<B> 0.5% $CuSO_4$ $5H_2O$ in 1% sodium or potassium tartrate.

<C> Alkaline copper solution: Mix 100 ml Reagent <A> with 2 ml Reagent <B>; discard after one day.

<D> Carbonate copper solution: Mix 100 ml of 2% Na_2CO_3 with 2 ml Reagent <B>; discard after one day.

<E> Diluted Folin Reagent: Dilute the Folin-Ciocalteau reagent to make it 1 N in acid.

<F> Protein standard: Dissolve bovine serum albumin in water to yield a solution that contains 2 mg protein per ml.

Procedure

1) Prepare dilutions of sample and standard <F> to 0.5 ml total volume.
2) Add 2 ml Reagent <C>; let stand 15 min at room temperature.
3) Add 0.2 ml Reagent <E>; mix immediately and let stand 45 min.
4) Read at 700 nm in a spectrophotometer

2. Coomassie Blue Protein Determination (3)

Reagents

<A> 20% Trichloroacetic acid

<B> Staining Solution: 0.25% w/v Coomassie Blue, 7.5% Acetic acid and 5% methanol.
<C> Destaining solution: 7.5% acetic acid, 5% methanol.
<D> Elution buffer: 0.12 N NaOH in 20% water: 80% methanol.
<E> 3 N HCl.
<F> Protein standard: 5 mg bovine serum albumin in 10 ml water.

Procedure

1) Number glass fiber filters (Whatman GF/A, 24 mm) near the outer edge. Use forceps to handle disks; do not touch with fingers.
2) Pipette sample of protein in solution (20 µl or less, containing 0.1-10 µg protein) onto the center of the filter and allow it to be absorbed completely (5-30 sec).
3) Plunge the filter into a beaker of Reagent <A>, 4° C in an ice bath.
4) Swirl the filters and the acid gently (vigorous stirring destroys the filters) for 5 min and then transfer them individually to another beaker containing Reagent <B> in an ice bath. Approximately 50 ml of this solution in a 400 ml beaker is sufficient to stain 30-40 filters.
5) Allow the filters to stain for 20 min with occasional gentle agitation to separate filters.
6) Transfer the filters to another beaker containing Reagent <C> in an ice bath and swirl for several min.
7) Decant the solution and wash the filters two more times using Reagent <C> with gentle agitation to remove most of the unbound dye.
8) Again cover the filters with Reagent <C>. Remove individual filters, place on Bradley filtering apparatus and wash several times under water pump suction until the blank filters are colorless.
9) Place the filters on several sheets of filter paper to drain. Cut out stained areas avoiding black markings and place in labeled 10 x 75 mm test tubes.
10) Add 1.2 ml Reagent <D>; vortex and allow to stand until all the dye is removed from the filter (generally 5 min).
11) Acidify the solution with 60 µl 3 N HCl.
12) Vortex, then centrifuge at 1700 rpm for 5 min in a Sorvall GLC-1 centrifuge.
13) Remove the supernatant solution carefully with a Pasteur disposable pipette and read the absorbance at 590 nm in a Gilford spectrophotometer.

NOTE: If necessary, the samples may be kept for 12

hr before measuring absorbance, but must be left in the dark and capped. They should be centrifuged just before reading to remove any glass fibers.

3. Bromosulfalein Protein Determination (4)

Reagents

<A> Bromosulfalein: Dissolve 0.25 g in 2 ml of 0.1 N NaOH.

<B> 1 N HCl: Dilute 8.6 ml of concentrated HCl with 91 ml water.

<C> 1 M Citric acid: Dissolve 10.5 g in 50 ml of water.

<D> Staining solution: Combine 20 µl of Reagent <A>, 2 ml of Reagent <B> and 0.5 ml of Reagent <C> mix by shaking.

<E> Wash solution: Mix Reagent <C>, Reagent <B>, and 1 N NaOH (1:2:2).

<F> Protein Standard: Bovine serum albumin. Dissolve 0.1 g per 10 ml water; use 0.5-10 µg range.

<G> Sodium deoxycholate: 10 mg/ml

Procedure

1) To the protein sample of 0.2 ml (in a 1.5-ml polypropylene centrifuge tube) add 200 µl 2 N NaOH (if dissolving a precipitated protein) add 20 µl of Reagent <G>. Incubate at room temperature for 90 min.
2) Vortex and add 50 µl of Reagent <D> and 400 µl of 1 N HCl. Vortex vigorously.
3) Centrifuge 5 min at 10,000 rpm in a Beckman Microfuge centrifuge.
4) Remove and discard the supernatant solution. Wash the precipitate with 1 ml of Reagent <E>.
5) Centrifuge again; remove and discard the supernatant solution.
6) Dissolve the precipitate in 1 ml of 0.1 N NaOH and determine absorbance at 580 nm in a spectrophotometer.

4. o-Phthalaldehyde (5)

Without Hydrolysis

Reagents

<A> Borate buffer: Dissolve 9.53 g of $Na_2B_4O_7 10\ H_2O$ in 500 ml water and titrate to pH 9.5 with 4 N NaOH; yields 0.05 M.

<B> Buffered Reagent: Mix 1.5 ml of o-phthalaldehyde solution (15 mg/1.5 ml ethanol) with 90 ml of borate buffer. Add 1.5 ml of a solution of 2-mercaptoethanol (7.5 µl/1.5 ml ethanol) and mix. The reagent is stable one day at room temperature.

<C> Protein standard: Bovine serum albumin
Dissolve 10 mg in 100 ml of 1 M NaCl

Procedure

1) Pipette Reagent <B> (1.75 ml) into a quartz fluorescence cuvette.
2) Add a sample containing from 0.1 to 25.6 µg of bovine serum albumin directly to the cuvette (add 1 M NaCl solution before protein addition so that final volume of solution is 2 ml and the final NaCl concentration is 0.125 M).
3) Mix the solution vigorously for 5 sec.
4) Illuminate the contents of the cuvette at 340 nm in a SPF-125 spectrofluorimeter and measure the emitted light at 455 nm.
5) Record the resulting fluorescence intensity with an OmniScribe recorder.

With Hydrolysis (6)

Reagent

<A> Dissolve 3.8 g $Na_2B_4O_7$ $10H_2O$ and 0.34 g NaOH in 100 ml water (pH 10.0).
<B> o-Phthalaldehyde reagent: combine
2 mM o-phthalaldehyde, 26.8 mg in 3 ml ethanol;
4 mM mercaptoethanol, 28 µl in 6 ml ethanol;
and 10 ml Reagent <A> to a final volume of 100 ml.
<C> 0.5 N NaOH (2 g/100 ml water)
<D> Alanine, as an amino acid standard.

Procedure

1) Pipette 50 µl of protein solutions (or alanine standards) into small soft glass test tubes, previously cleaned in 6 N HCl at 100°C for 4 hr.
2) Add 50 µl of 12 N HCl
3) Heat for 5 hr at 110°C (in sealed tube), 50 µl of the solution was drawn for analysis.
4) Wash the fluorescence cuvette with o-phthalaldehyde reagent until a low background is reached.
5) Pipette 1 ml of Reagent <B> into the fluorescence cuvette.
6) Pipette 50 µl of protein hydrolysate and 5 µl of 6 N NaOH into the cuvette. Use vigorous mixing (20 shakings) to achieve homogeneity of solution.
7) Measure fluorescence as in steps 4 and 5 of the Without Hydrolysis Procedure (above).

B. DNA Determinations

1. Diphenylamine DNA Determination (7)

Reagent

<A> DNA 50 µg/ml: Weigh 5.0 mg DNA (salmon sperm) and dissolve it in 100 ml of 0.145 M NaCl.
<B> 0.145 M NaCl: Dissolve 8.48 g NaCl in 1 liter of deionized water.

<C> 4% Diphenylamine in glacial acetic acid: Dissolve 4 g of diphenylamine in 91.4 ml of glacial acetic acid.

NOTE: Light-sensitive--prepare on day of use and store in brown glass bottle.

<D> 20% $HClO$: Dilute 17 ml of 70% $HClO_4$ with 83 ml water.

<E> CH_3CHO, 1.6 mg/ml: Mix 1 ml CH_3CHO (16 mg/ml) with 9 ml water. KEEP REFRIGERATED. Or use 10 µl of paraldehyde per 100 ml of Reagent <C>.

<F> Amyl acetate (pentyl acetate), reagent grade.

Procedure

1) Add various concentrations of DNA (1-50 µg) in Reagent <B> (total volume 1 ml) to 1.0 ml of Regent <D>. Assay is triplicated for each concentration. Prepare unknown in the same way.
2) Add 2 ml Reagent <C> and 100 µl of Reagent <E>.
3) Vortex.
4) Incubate for 1 hr at 56°C (water bath).
5) Cool tubes to room temperature in tap water.
6) Add 0.5 ml Reagent <F> to each tube.
7) Vortex.
8) Centrifuge at 1600 rpm for 1 min at room temperature in a Sorvall GLC-1 centrifuge.
9) Remove the organic layer with a Pasteur disposable pipette and transfer to a clean empty test tube.
10) Repeat steps 6-9 twice, placing the extract in the same matched tubes each time.
11) Read adsorption at 595 nm in a 1-ml cuvette in a Gilford spectrophotometer.

2. 3,5-Diaminobenzoate DNA Determination

a. Spectrophotometric (8)

Reagent

<A> 1 N $HClO_4$: 45 ml of 70% $HClO_4$ + 455 ml of water.

<B> 0.6 N $HClO_4$: 27 ml of 70% $HClO_4$ + 473 ml of water.

<C> 3,5-Diaminobenzoic Acid reagent: Immediately before use disolve 300 mg of 3,5-diaminobenzoic acid dihydrochloride in 1 ml water (good for 10 assays).

<D> DNA Standard: Dissolve 250 mg of DNA in 100 ml of 1 N NH_4OH.

Procedure

1) Treat samples and DNA standards with 0.1 ml of Reagent <A> for 10 min at room temperature in 10 x 75 mm glass test tubes.
2) Add Reagent <C> (0.1 ml) to each tube.
3) Cap the tubes and incubate in a 60°C

heating block for 30 min.

4) Add 1.8 ml Reagent <B> to each tube and mix the solution in a Vortex mixer.

5) Transfer the reaction mixture to a 1-ml cuvette and read absorbance at 420 nm in a spectrophotometer.

b. Fluorimetric (9)

Reagent

<A> 3,5-Diaminobenzoic acid (purified):

1. Add 2.0 ml of 4 N HCl at 5°C to 0.6 g of DBA.
2. Agitate the mixture with Vortex mixer until the solid is dissolved.
3. Transfer the solution to a 12-ml conical centrifuge tube containing 200 mg of Norit A and mix by drawing it in and releasing it from a Pasteur pipette.
4. Centrifuge the mixture at 3000 rpm for 5 min at room temperature in a Sorvall GLC-1 centrifuge.
5. Remove the supernatant solution with a Pasteur pipette and transfer to a second conical tube containing 20 mg of Norit A.
6. Repeat steps (3) and (4) four more times.
7. Remove the supernatant solution with a Pasteur pipette and deposit it in a conical tube. Centrifuge again to remove the remaining Norit A.

<B> DNA standard: Prepare by weighing 16.0 mg of DNA and dissolving in 10 ml of 1 N NH_4OH.

Procedure

1) Wet Metricel filters (Alpha-6 Cellulose, Gelman) with water on a Bradley filtration apparatus and wash twice with 0.15 ml of 0.6 N (10%) trichloroacetic acid at 5°C. Repeat this washing using 0.15 ml of 5°C ethanol:water (2:1) and 0.15 ml of 60°C ethanol:water (2:1).

2) Remove the filters from the filtration apparatus and place in BEEM polyethylene capsules. Add standards and samples (10 µl) to the filters and dry at 20°C (usually overnight).

3) Add to each filter 100 µl of Reagent <A>. Seal the capsules and incubate in a 60°C water bath for 30 min.

4) Cool the capsules at 20°C for 5 min; then add 1.0 ml of 1 N HCl to each capsule (0.5 ml at a time), transfer the solution to a cuvette

and read fluorescence in a SPF-125 spectrofluorimeter (Hg-Xe lamp). Excitation, 405 nm; emission, 520 nm.

3. Ethidium Bromide DNA Determination (modified from 10-13)
   Reagents
   <A> HEPES buffer: 0.05 M, pH 7 (1.2 g in 100 ml) and 0.012 M NaCl.
   <B> Ethidium bromide: 25 μg/ml or 1.5 μg/ml (depends on DNA concentration).
   <C> Pronase: 80 μg/ml in HEPES buffer prepared fresh daily.
   <D> DNA: 25 μg/ml.
   <E> Ribonuclease: 20 μg/ml in HEPES buffer, heated to 80°C for 10 min.
   Procedure
   1) Incubate a mixture of sample (0.5 ml), 0.5 ml Reagent <C> 0.5 ml Reagent <E>, and 0.5 ml Reagent <A> 20 min at 37°C. Use controls leaving out reagents and substituting buffer as indicated in Table 1.
   2) Add 0.5 ml of Reagent <B>. The maximum fluorescence is achieved in 1 min and remains for at least 1 hr.
   3) Determine fluorescence in SPF-125 spectrofluorimeter with excitation at 546 nm and emission at 590 nm.
   4) Use fluorescence of reagents less sample and the inherent fluorescence of the sample to correct the measured value.

4. 4',6-Diamidino-1-phenylindole DNA Determination (14)
   Reagent:
   <A> 4',6-Diamidino-1-phenylindole 2 HCl. Dissolve 1.0 mg DAPI in 250 ml water.
   <B> DNA: dissolve calf thymus DNA at 250 μg/ml in 0.012 M NaCl.
   <C> 0.012 M NaCl: dissolve 0.70 g of NaCl in 1 liter of water.
   <D> 0.05 M HEPES buffer: dissolve 0.12 g HEPES in 10 ml of water.
   Procedure
   1) Use samples and standards in a volume of 0.8 ml.
   2) Add 0.1 ml Reagent <D>.
   3) Add 0.1 ml Reagent <A>.
   4) Measure fluorescence in a SPF-125 spectrofluorimeter immediately after mixing. Excitation, 355 nm and emission at 454 nm.
      Instrument parameters
      #5 slit arrangement;
      multiplier, 0.001 or 0.003;

TABLE 1. COMPOSITION OF REACTION MIXTURES FOR DNA DETERMINATION WITH ETHIDIUM BROMIDE

Reaction Mixture	Designation	Components Reagent	Volume (ml)
1	Standard	<A>	1.0
		<B>	0.5
		<C>	0.5
		<D>	0.5
2	Blank I	<A>	1.5
		<B>	0.5
		<C>	0.5
3	Blank II	<A>	2.5
4	Sample (DNA + RNA)	<A>	1.0
		<B>	0.5
		<C>	0.5
		Sample	0.5
5	Sample (DNA)	<A>	0.5
		<B>	0.5
		<C>	0.5
		Sample	0.5
		<E>	0.5
6	Sample (background correction)	<A>	2.0
		Sample	0.5

lamp, xenon; and
cooling water, 24°.

C. RNA Determinations

1. Orcinol RNA Determination (15)

Reagents

<A> 0.004 M $CuCl_2$ H_2O in conc. HCl.
<B> Conc. HCl.
<C> Orcinol reagent (must be prepared fresh):
a) dissolve 200 mg orcinol in 10 ml Reagent <B>
b) add 10 ml of Reagent <A>
c) dilute to 100 ml with conc. HCl
<D> RNA standards: 2.0 mg yeast RNA/100 ml water.

Procedure

1) Dilute sample to a total volume of 1 ml using water.
2) Add 1 ml Reagent <C>; vortex thoroughly.
3) Heat in boiling water bath 40 min with a marble on top of each tube.
4) Cool in running water.
5) Extract colored product:
a) add 2 ml isoamyl alcohol and vortex immediately.
b) vortex all tubes again immediately before placing in centrifuge.
c) centrifuge at 1700 rpm for 5 min in a Sorvall GLC-1 centrifuge.
6) Draw off approximately 1 ml of alcohol layer and read absorption at 675 nm in a spectrophotometer.

NOTE: All the color is not extracted.

2. Ethidium Bromide RNA Determination (modified from 10-13)

Reagents:

<A> HEPES buffer: 0.05 M pH 7, 1.2 g in 100 ml of 0.012 M NaCl.
<B> Ethidium bromide: 25 µg/ml
<C> Pronase: 80 µg/ml in HEPES buffer prepared fresh daily.
<D> RNA: 25 µg/ml

Procedure:

1) Incubate sample (0.5 ml), 0.5 ml of Reagent <C>, and 1 ml Reagent <A> together 20 min at 37°C.
2) Add 0.5 ml of Reagent <B> . The maximum fluorescence is achieved in 1 min and remains for at least 1 hr.
3) Determine fluorescence in a SPF-125 spectrofluorimeter. Excitation, 546 nm; emission 590 nm.
4) Use fluorescence of reagents without sample and the inherent fluorescence of the sample (without Reagent <B>) to correct the measured value.

The following formula is used to calculate the amount

of RNA present in a mixture of DNA-RNA.

$$A_{RNA} = \frac{A_{std} \quad (f_D - f_E)}{0.46 \qquad (f_A - f_D)}$$

where A_{DNA} = Amount of DNA per mixture (μg)

A_{std} = Amount of standard DNA per mixture A (μg)

f = fluorescence intensity (units)

A_{RNA} = Amount of RNA per mixture (μg)

The factor 0.46 is empirically derived from the ratio of fluorescence for ethidium RNA/DNA

D. Organic Phosphate Determination (16, 17)

1. Orthophosphate Determination

Note: This procedure is the basis for phosphate both organic and inorganic.

Reagents:

<A> Mixed reagent: Dissolve 2.12 g of Na_2MoO_4 $2H_2O$ in 150 ml distilled water. Add 20.8 ml of concentrated H_2SO_4 slowly with stirring. Cool to room temperature. Dissolve 0.0668 g of potassium antimony tartrate in 20 ml of water. Add the potassium antimony tartrate solution to the acidic sodium molybdate solution (slowly with stirring) and dilute to 250 ml. The reagent is stable for several weeks at room temperature.

<B> Ascorbic acid: Prepare a 1% solution daily.

<C> Acetophenone-chloroform: Add 10 ml $CHCl_3$ to 90 ml acetophenone and pass through an alumina column.

<D> Sulfuric acid solution, 11 N: Slowly add 310 ml concentrated H_2SO_4 to 600 ml distilled water. When cool, dilute to one liter.

<E> Ammonium persulfate.

<F> Sodium sulfate, anhydrous.

<G> Stock phosphorus solution: Dissolve in distilled water 0.2197 g of potassium dihydrogen phosphate. KH_2PO_4 which has been dried in an oven at 105°C. Dilute the solution to 1000 ml; therefore, 1 ml = 50 μg P.

<H> Standard phosphorus solution: Dilute 1.0 ml of Reagent <G> to 10 ml with distilled water; then, 1 μl = 5 ng P. Using Reagent <H>, prepare

standards in 200 ml solution ranging from 0 to 32 ng of P per ml.

<I> Sodium hydroxide, 1 N: Dissolve 40.0 g NaOH in 600 ml distilled water. Cool and dilute to 1 liter.

NOTE: Store this solution in a plastic container because a high background results when the alkali is stored in a glass container.

Procedure

1) Using Table 2 as a guide, place the appropriate volume of sample into a separatory funnel, add the indicated volume of Reagent <A> and 2 ml of Reagent <B>.
2) Allow 20 min for complex formation and then extract with the indicated volume of Reagent <C> by shaking vigorously for 1 min.
3) After the layers have separated, transfer as much of the bottom layer as possible to a 15-ml centrifuge tube.
4) Add about 1 g anhydrous Reagent <F> and centrifuge briefly.
5) Measure the absorbance of the organic phase at 700 nm in a 1-cm cell against a blank. A reagent blank should be used throughout the procedure.

2. Organic Phosphate Determination by Hydrolysis

Procedure

1) Add 4 ml of 11 N H_2SO_4 solution to a sample in a 400-ml beaker.
2) Add 1.6 g of ammonium persulfate.
3) Boil gently on a hot plate for 30-40 min until a final volume of about 40 ml is reached. Do not allow sample to go to dryness.
4) Adjust the pH of the sample to 7.0+0.2 with 1 N NaOH using a pH meter. Cool and dilute the sample to 200 ml.
5) Determine total phosphate as outlined above.
6) Compute organic phosphate as total phosphate - orthophosphate.

E. Coenzyme Determination

1. Pyridine Nucleotides $NAD(P)^+$

a. Spectrophotometric or Fluorimetric Assay

Reduced pyridine nucleotides (NADH and NADPH) are measured either spectrophotometrically at 340 nm or fluorometrically with excitation at 340 nm, 1-mm slit and emission at 455 nm, 2-mm slit. Sensitivity (high voltage) of the spectrofluorimeter is set at maximum for all measurements. Standard NADH

TABLE 2. GUIDELINES FOR PHOSPHATE ANALYSIS

Range (P conc.,PPB)	Sample Volume (ml)	Reagent <D> (ml)	Reagent <C> (ml)
3-40	200	10	5
10-100	200	10	10
40-400	100	5	10
100-1,000	50	2.5	10

solutions and 5 mM quinine sulfate are used to monitor variations in lamp intensity (20).

b. Bioluminescent Assay--A NADH test kit from Lumac Co. can also be used for luminescence determinations of NADH. The luciferase reagent contains 9.7 nmol FMN, 10 µg of an unspecified aldehyde, and an unknown amount of enzyme preparation in 0.1 M phosphate buffer, pH 6.9. The sample (0.1 ml) is injected into 0.4 ml reagent and the luminescence integrated over 5 min with the Packard Pico-Lite photometer (32).

c. Enzyme Cycling-- (see Figure 1)
Amplification of $NADP^+$ and NAD^+ by enzyme cycling is carried out by the procedure of Lowry and Passonneau (28) as modified by Chi et al. (29). The procedure has two steps:
First, $NAD(P)^+$ is cycled for some time, usually 1 hr, after which the reaction is stopped by heat; and second, the product of one of the cycling enzymes, 6-phosphogluconate, is measured. The cycling reagent consists of 5 mM α-ketoglutarate, 10 mM ammonium acetate, 0.1 mM 5'ADP, 1.0 mM glucose 6-phosphate, 0.072 U glutamate dehydrogenase, and 0.5 U glucose 6-phosphate dehydrogenase in 100 mM Tris acetate, pH 8.0. The amounts of GDH and G6PDH used depend upon the specific activities of the enzyme preparations used. Optimal amounts of enzymes used in the cycling reagent are determined by the procedure of Lowry and Passonneau (20). The gluconate reagent contains 0.1 mM EDTA, 30 mM ammonium acetate, 5 mM $MgCl_2$, 30-100 µM $NADP^+$, and 25-100 mU 6-phosphogluconate dehydrogenase in 40 mM Tris-HCl, pH 8.1.

The procedure for enzymatic cycling of pyridine nucleotides is as follows. To a 50-µl sample in a 1.5 ml plastic tube add 100 µl cycling reagent with a Brinkmann Dilutor Dispenser. The samples are incubated 30-120 min, depending on the amounts of pyridine nucleotides in the sample, at 37°C in a water bath. The reaction is stopped by placing the tubes in a 100°C water bath for 2 min. The tubes are cooled 10 min at 4°C and 1.0 ml gluconate reagent added. After a 30-min incubation at 37°C the fluorescence intensity of NADPH is determined in a spectrofluorimeter (exciation, 340; emission, 455 nm).

2. Flavins
Reagents:

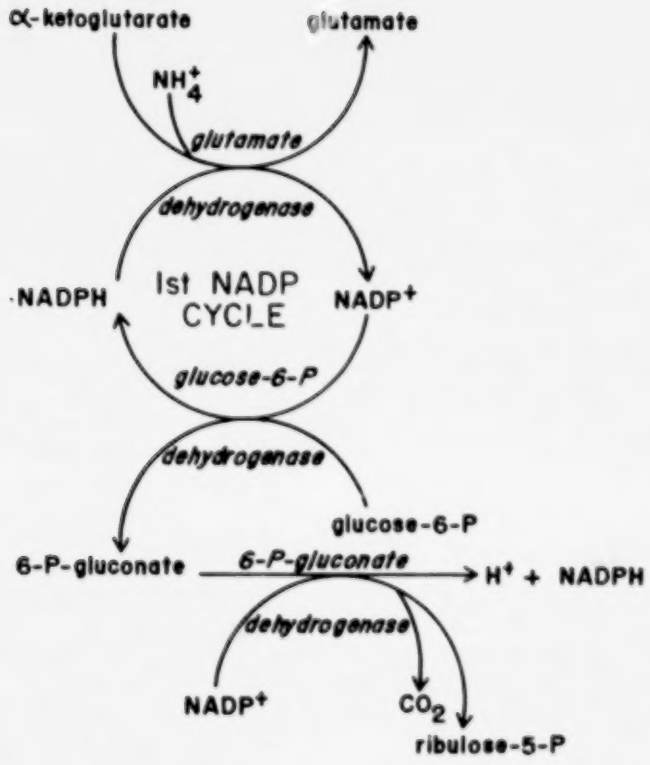


Figure 1. Reaction scheme for the enzymatic cycling of $NADP^+$. The sensitivity in determining the amount of a cofactor can be amplified through the catalytic use of that cofactor. The cofactor is utilized in one reaction and regenerated in a coupled reaction.

<A> $NaBH_4$: 14.8 mg/ml in 0.4 N KOH
<B> 0.4 N KOH: 2.58 g KOH/100 ml water
<C> $PdCl_2$: 0.17 mg/ml in 0.4 N HNO_3
<D> 0.4 N HNO_3: 2.53 ml of 15.8 N HNO_3, dilute to 100 ml
<E> 0.4 M Tris: 4.32 g Tris/100 ml water, adjust pH to 6.75
<F> Luciferase, Sigma, Bacterial, purified, 2 mg/ml
<G> Dodecanal — 0.015 mg/ml water, suspended using sonification.

Procedure:

1) Mix 1 ml of $PdCl_2$ solution with 1 ml of sample and let stand for 5 min.
2) Add 1 ml of $NaBH_4$ to reduce FMN.
3) Add 1 ml of Reagent <E> to the above solution.
4) Within 10 min after the borohydride addition inject 0.2 ml of sample into a cuvette containing 0.1 ml of Reagent <F> and 0.1 ml of Reagent <G>.
5) Measure the light production by both peak height and integration for 30 sec in the Pico-Lite photometer.

Extraction Procedure (see p. 25).

3. Iron Porphyrins (modified from 34-39)

a. Determination of Hemoglobin--Luminol chemiluminescence is used to quantitate hemoglobin by injection of 0.2-ml samples into 0.5 ml of luminol reagent containing 0.25 mM luminol, 10 mM EDTA, and 290 mM hydrogen peroxide in 50 mM phosphate buffer, pH 11.6. The SAI Model 3000 photometer is set for 0.5 sec delay and 10 sec count with the sensitivity at 7.00. The light intensity in the assay is attenuated with aluminum disks with different-sized holes so that the range of hemoglobin concentration measured can be extended.

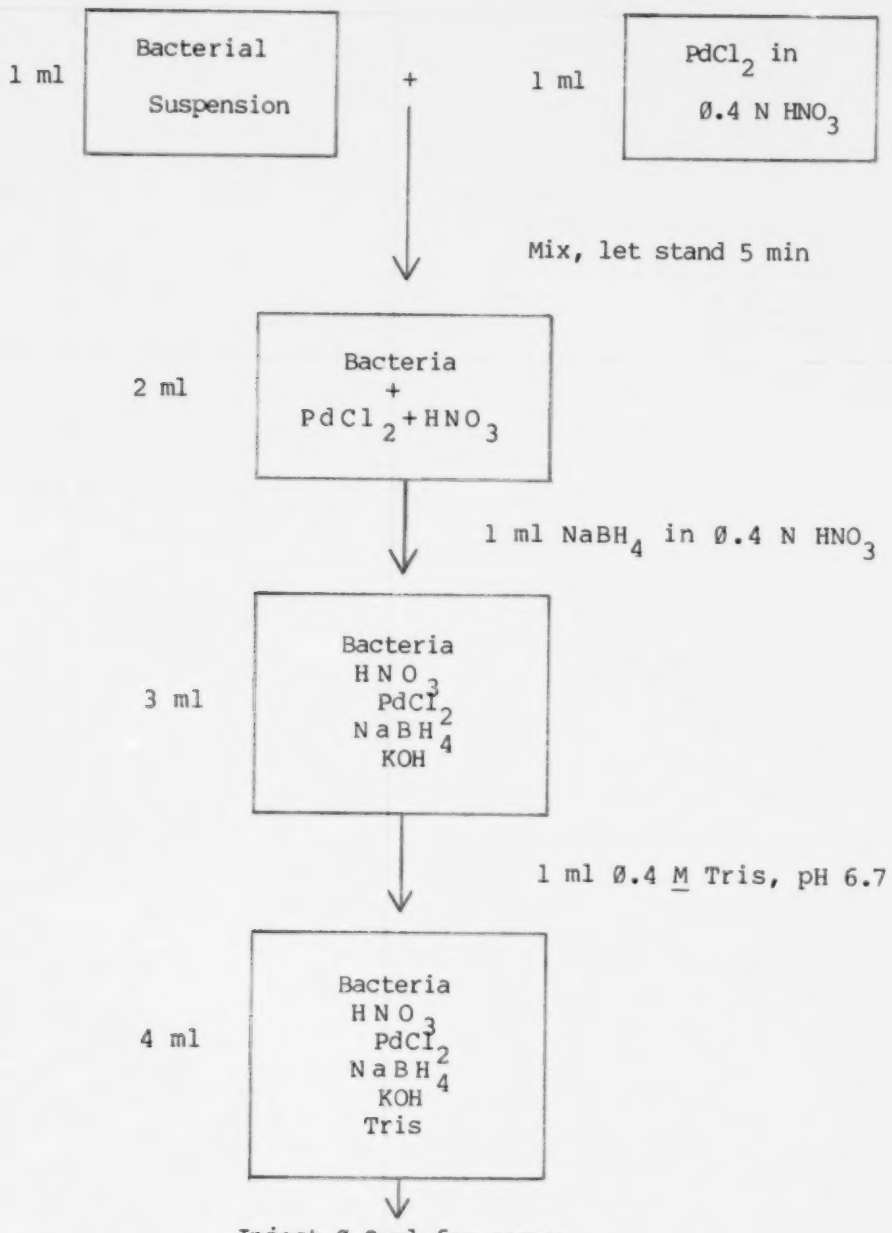
4. ATP

a. Measurement of ATP by Enzyme Cycling (28)
ATP is measured by the hexokinase reaction with glucose to form glucose 6-phosphate. The glucose 6-phosphate is oxidized by glucose 6-phosphate dehydrogenase to produce NADPH. The NADPH signal is amplified by enzyme cycling and ultimately measured as described previously in section E.1.c.

b. Firefly Luciferase Assay (41-44)
Reagents:
<A> Luciferase

EXTRACTION and REDUCTION

of INTRACELLULAR FLAVINS



1) Du Pont

i. Dissolve luciferin-luciferase powder contained in one vial (100-140 mg dry weight) by adding 3 ml of 0.05 M Tricine buffer containing 0.01 M $MgSO_4$, 0.001 M DTT, and 0.001 M EDTA (referred to as 0.05 M complete buffer).

ii. Label the vial, wrap in foil, and age overnight (16-18 hr) at 4°C. In this form the luciferase can be used for 7-10 d without significant loss of sensitivity.

iii. The enzyme can be prepared and aged 2-4 hr at room temperature (20-25°C) prior to using. However, at 24°C the inherent light emission (without addition of ATP) is reduced from 48,400 counts to 840 counts in 5 hr. Additional reductions to 580 and 200 counts occur within 24 and 48 hr of aging, respectively.

iv. The enzyme is used at room temperature (20-25°C).

2) Sigma Type IV Luciferase

i. Weigh a sample (usually 1-2 mg) of Sigma Type IV luciferase and dissolve in 200 µl of 10% $(NH_4)_2SO_4$, pH 7.8. After 10 min at room temperature add 1-2 ml of complete 0.05 M Tricine buffer and 400 µg of BSA.

ii. Wrap the glass vial in foil and age overnight at 4°C.

iii. The next morning add sufficient complete 0.05 M Tricine buffer to yield a stock solution of 0.5 µg/µl and BSA (100 µg/ml). Store this preparation at 4°C wrapped in foil; it is stable for 2 wk.

iv. Prepare a working solution of Sigma Type IV luciferase by diluting 100 µl of the stock solution with 400 µl of complete 0.05 M Tricine buffer. The working solution (0.1 µg luciferase per µl) is made fresh daily.

<B> Buffer

1) Tricine

i. 1 M stock solution

Weigh 17.92 g of Tricine and dissolve in 70-80 ml of water (see below) in a 100-ml beaker; cover with Parafilm and stir with a magnetic stirrer

(10-30 min is usually required). Add 1.5-2 ml 10 N NaOH and test pH (should be 7.6-7.8). Add sufficient water to yield 100 ml total volume. Store in a sterile prescription bottle at 4°C.

ii. Complete 0.25 M (used in assay, 100 µl/ml reaction vol).
25 ml of 1 M Tricine
5 ml of 1 M $MgSO_4$
5 ml of 0.1 M DTT
5 ml of 0.1 M EDTA
60 ml of water (see below)
10-20 µl of 10 N NaOH is used to adjust pH to 7.8
Store frozen in 10-20-ml aliquots in 1-oz screw cap prescription bottles. (A high blank, when all other reagents are fresh, can usually be traced to contaminated buffer). Use at room temperature.
Store at 4°C for not more than 2 wk.

iii. Complete Tricine 0.05 M (used to dilute enzyme preparations and for extraction).
5 ml of 1 M Tricine
1 ml of 1 M $MgSO_4$
1 ml of 0.1 M DTT
1 ml of 0.1 M EDTA
92 ml of water
Adjust pH to 7.8 if needed and store at 4°C.

iv. 0.5 M Tricine (used to wash cells and suspend cells for extraction)
5 ml of 1 M Tricine
95 ml of water

v. 0.05 M Tricine for extraction
5 ml of 1 M Tricine
1 ml of 1 M $MgSO_4$
2 ml of 0.1 M EDTA
92 ml of water

<C> Magnesium Sulfate 1 M
1) Weigh 24.65 g $MgSO_4$ $7H_2O$ and dissolve in 100 ml of water.
2) Dispense into 1-oz prescription bottles and freeze.

<D> Cleland's Reagent (DTT) 0.1 M
1) Weigh 0.386 g and dissolve in 25 ml water.
2) Cap tightly and store at 4°C.
3) Prepare fresh every month.

<E> Ethylenediaminetetraacetic acid disodium salt

1) Weigh 3.722 g EDTA and dissolve in 100 ml water.
2) Store at 4°C.

<F> Water

1) Use glass-distilled water that has been pressure filtered (Millipore, 0.22-μm pore size) and autoclaved (as for tissue culture) to prepare all solutions used in the assay.
2) To prepare extracting solutions and washing solutions, use water that has been glass distilled and autoclaved.

<G> Bovine Serum Albumin (1 mg/ml)

1) Weigh 50 mg of bovine serum albumin; put 40 ml of water into a 100-ml beaker; tap bovine serum albumin onto top of water and let stand until it dissolves (stirring should be kept to a minimum to prevent denaturation); add water to make volume to 50 ml.
2) Store frozen in small aliquots.
3) Add BSA to Sigma Type IV and other crystalline luciferase preparation to yield 100 μg/ml in stock solutions. (BSA is already present in the Du Pont and several other commercial preparations.)

NOTE: Sterility is the key to a reagent's long shelf life.

<H> Adenosine Triphosphate (ATP) -- The disodium salt of equine muscle ATP obtained from Sigma Chemical is used.

1) Prepare a stock solution of 1 mg/ml (approximately 2 mM) by weighing 25 mg of ATP and dissolving in 25 ml of sterile glass-distilled water.
2) Serial dilutions are made and stored frozen in sterile glass vials or polypropylene snap-cap vials. These are color coded as shown below:

Color	Concentrations (approximate)	ng/ml
Pink	2 mM	1,000,000
Red	200 μM	100,000
Green	20 μM	10,000
Yellow	2 μM	1,000
Blue	200 nM	100

3) ATP standards at concentrations of 1×10^4

ng/ml (green) or greater are stable for at least 1 y when stored frozen under sterile conditions. Standards of 100 or greater ng/ml are stable for 3 d at 4° C if sterility is maintained. Standards of less than 100 ng/ml (blue) should be prepared fresh daily and kept on ice.

4) For very dilute solutions the standard ATP is prepared and diluted in 0.025 M Tricine containing 0.001 M EDTA.

<I> Luciferin

1) Source

Calbiochem A grade, luciferin prepared by Berlin and Radhakrishna of the OSU Dept. of Chemistry, or Boehringer-Mannheim.

2) Solution Preparation

i. Pure luciferin is stored at 4°C in tightly sealed vial wrapped in light-proof paper and placed with desiccant in a brown bottle wrapped in Parafilm and sealed with tape.

NOTE: Luciferin should be protected from light at all times.

ii. Prepare stock solutions of luciferin at 5 mg/ml concentrations. For this purpose weigh 100 mg of luciferin and dissolve in 18 ml of sterile water; stir magnetically in ice bath or cold room (may take 2 hr); dilute to 20 ml; dispense the solution into glass vials (1-2 ml/vial) -- displace the air with N_2, wrap in foil, label and store frozen.

iii. Working solutions are prepared by diluting 100 µl of stock with 400 µl water to yield 500 µl of 1 mg/ml luciferin. Mix, use and store in brown bottle sealed with Parafilm. Working solution if kept on ice is stable for one working day--it can be stored frozen, thawed and used again twice.

3) Stability

i. Working solutions (1 mg/ml) are stable for 1 month stored frozen under N_2.

ii. Stock solutions (5 mg/ml) are stable for at least a year.

Procedure

1) Equipment and Its Operation

i. Photometer

a. Description - A JRB-integrating photo-

meter Model 3000 marketed by SAI Technology and equipped with variable time delay and integration options and the peak height kinetic kit is used. A Houston Instrument (Omni-Scribe Recorder Model A511 5-5) is also used. The instrument is routinely left with power switch ON and with the SENSITIVITY set at 0.00. A voltage regulator (Cornell Dubilier IF 18) is inserted in line to modulate power surges. The polished reflective surface of the sample holder is cleaned every two wk with methanol and lens tissue.

b. Use -- If instrument maintains zero reading (dark current), proceed with experiment. Check (^{3}H) standard at beginning and end of experiment to detect any instrument instability. Since there are no temperature regulation capabilites, the instrument is operated at room temperature (in an air-conditioned room).

ii. Pipettes

Gilson Pipetman P-20, P-200, and P-1000 and the Eppendorf 50 µl automatic pipettes are used to dispense reagents for the assay. Disposable tips are used.

iii. Glassware

a) Soak dirty glassware in phosphate-free detergent (Detergent "1.2.2" from Curtin Scientific Co., Houston, TX.)
b) Wash glassware with hot soapy water and brush.
c) Rinse with tap water.
d) Air dry.
e) Soak overnight (minimum) in 6 N HCl.
f) Rinse several times with tap-distilled water and twice with sterile glass-distilled water.
g) Air dry.
h) Cover with foil and autoclave.
i) Store in closed cabinet in the dark.

iv. Reaction Vessels

Biovials obtained from Beckman (3 ml polypropylene vials) are used.

2) The Assay

i. Mixtures

a) Du Pont

0.025 M Tricine, pH 7.8

5 mM $MgSO_4$
0.5 mM EDTA
0.5 mM DTT
400 µg luciferase and luciferin (dry weight)
Total volume, 1 ml

b) Sigma Type IV
0.025 M Tricine, pH 7.8
5 mM $MgSO_4$
0.5 mM EDTA
0.5 mM DTT
50 µg luciferin
2 µg luciferase
100 µg BSA (if not included in enzyme stock solution)
Total volume, 1 ml

ii. Protocol

a) Instrument setting
Zero 4.30 (usual)
Sensitivity 7.00
Delay 15 sec
Assay 60 sec
Integration mode
30 min warm-up time

b) Room setting (room dark)
Because of the adverse effect of light on luciferin and because fluorescent lights excite glass vials (if used instead of polypropylene) for light emission, the assay is done in a darkened room.

c) Order of addition
(1) 100 µl complete buffer
(2) Add water sufficient to make the total reaction volume 1 ml when all components are added.
(3) ATP or sample (100 µl is used)
(4) 50 µl luciferin (if required); mix
(5) 20 µl of Sigma Type IV luciferase
Simultaneously start time delay by pressing the foot switch upon addition of luciferase.

d) Operation
(1) Swirl the contents of the vial for 8-10 sec; insert vial into the chamber, close the chamber lid, open shutter, and accumulate counts for 1 min.
(2) Record the counts from the digit-

al display; close shutter, reset, remove used vial, and proceed to the next sample (approximately 2 min required per sample).

3) Use of Phenol Red

The pH at which the assay is done is critical; phenol red (phenolsulfonphthalein) is an indicator whose useful range of 6.8-8.2 (yellow-red) spans the area of interest (pH 7.6 orange). We use 100 µl of 0.1% phenol red (dissolved in 95% ethanol and and diluted in water). Phenol red inhibits the reaction 50%; therefore, the pH is best verified after the assay.

4) ATP Standard

The range of standards used are: 0.05-100 ng for Du Pont luciferase and 0.01-5 ng for Sigma Type IV luciferase. To correct for any inhibitory agents present in the samples a known amount of ATP, such as 1 ng, is added to another sample, and the light production is determined. By appropriate comparison among the sample itself, the sample spiked with known amounts of ATP, and the response to the known ATP in the regular assay system, a correction can be made for inhibition.

5) Calculations

If the inherent light value (background) is less than 1% of the light production by the sample, no correction is made; otherwise the inherent light (blank value) is subtracted from the observed light production. A standard curve of log light produced (cpm) versus log of ATP concentration is plotted; the unknown ATP amount is determined from this curve. Usually samples are assayed at least in triplicate and the standard curve points are assayed in quintriplicate.

Extraction Procedures

1) Boiling Tricine

Solution preparation:

0.05 M Tricine containing 0.01 M $MgSO_4$ and 0.001-0.002 M EDTA, pH 7.6

Procedure:

a. Heat 4.5 ml of buffer in rapidly boiling water bath 5 min.
b. Inject 0.5 ml of cell suspension (in buffer) into buffer.
c. Boil 2 min.

d. Chill on ice 10 min.
e. Quick freeze in Dry Ice/acetone unless samples are to be assayed same day.
f. Assay 100-500 ul of extract depending on activity.

Note on cell preparation:
Cells in the log-growth phase are harvested by centrifugation and washed 2 X with 0.05 M Tricine or minimal medium to remove substances which inhibit the luciferase-luciferin assay.

2) Dimethyl Sulfoxide
Prepare 90% DMSO by diluting with 0.1 M Tricine.
For cells:
a. Pipette 1 ml of cell suspension into 2 ml 90% DMSO.
b. Vortex vigorously 1 min.
c. Centrifuge, decant the supernatant solution.
d. Dilute the supernatant solution 1:10 or 1:100 with 0.05 M Tricine.
e. Assay 100-500 µl depending on activity.

For soil:
a. Weigh 1 g of soil into test tube.
b. Add 10 ml 90% DMSO.
c. Vortex vigorously 1 min.
d. Centrifuge and decant the supernatant solution.
e. Dilute 1:10 (or 1:100 if necessary).
f. Assay 500 µl.

3) Sulfuric Acid
a. Prepare 1.5 N H_2SO_4 (41.5 ml of concentrated acid (36^4 N) in 1000 ml water).
b. Grind, sieve, and stir soil sample to obtain a homogeneous sample.
c. Weigh 1 or 2 g into a sterile 18 x 150 mm test tube.
d. Add 10 ml of the 1.5 N H_2SO_4.
e. Treat in the Polytron 1 min.
f. Shake on wrist action shaker 30 min at 4° C.
g. Centrifuge to remove soil
h. Decant the supernatant solution into a sterile test tube.
i. Neutralize extract:
Either Dilute 1:10 with 0.1 M Tricine

pH 9 and add 5 N ethanolamine to obtain pH 7.5.

or Treat 2 ml of extract with 4 ml 0.5 M Alamine/Freon overnight; centrifuge and dilute 0.1-0.2 ml of the aqueous layer with 0.1 M Tricine.

j. Assay 100 µl of sample.

F. Enzymes and Enzyme System Determinations

1. Adenylate Kinase

Adenylate kinase activity is measured by quantitation of ATP produced. The ATP generated by incubation of ADP with enzyme samples is measured using a coupled-enzyme assay (18), firefly luciferase (19), and enzymatic cycling (20). The adenylate kinase reagent contains 0.5 mM ADP and 5 mM $MgCl_2$ in 50 mM Tris-HCl buffer, pH 7.5.

a. Kinetic Assay-- The reagent components for the coupled-enzyme assay of ATP are added to the adenylate kinase reagent so that NADPH production is equivalent to the ATP generated by the adenylate kinase. The combined reagent (0.9 ml) is added to 0.1 ml samples and kinetic determinations are made spectrophotometrically at 37°C using a chart recorder.

b. Incubation Assay--Alternatively the mixture is incubated one hour at 37°C and NADPH quantitated fluorimetrically as discussed for pyridine nucleotide measurements. (see E.1.c).

2. Alkaline Phosphatase

a. Kinetic Assay--The continuous or kinetic assay for alkaline phosphatase is performed by the method of Malamy and Horecker (21). The substrate was 1.0 mM disodium p-nitrophenyl phosphate in 1.0 M Tris-HCl, pH 8.0. The sample (0.1 ml) is added to 0.9 ml reagent in a 1.0-ml cuvette at 37°C and the change in absorbance at 410 nm measured with a chart recorder. The minimum rate detectable is an absorbance change of about 0.005/min and activities are reported as A/min.

b. Incubation Assay--Two incubation assays can be used. The first uses a 1-hr incubation with 6.6 mM disodium p-nitrophenyl phosphate in 0.6 M Tris-HCl, pH 8.2. A 0.1-ml sample is mixed with 0.9 ml reagent and the solution incubated in a 38°C water bath for 1 hr. The alkaline phosphatase is measured by the increase in absorbance at 410 nm caused by the p-nitrophenol released in the reaction. A long-incubation assay is used to measure very small amounts of enzyme activity. Disodium p-nitrophenyl phosphate (0.0135 M in 1.0 M Tris-HCl, pH 8.0) is mixed 1:1 with sample (0.5 ml of each) and the mixture incubated at 37°C for up to four days. Enzyme activity was determined spectrophotometrically as in the 1-hr incubation assay (22).

3. Catalase (23)

a. Kinetic Assay--Catalase activity is measured by the disappearance of hydrogen peroxide which is quantitated spectrophotometrically at 240 nm with a chart recorder. Samples (2.0 ml) are mixed with 1.0-ml portions of 0.053 M H_2O_2 in 0.01 M phosphate buffer, pH 7.0, in a 3-ml cuvette. This is placed in a spectrophotometer whose sample chamber is kept at 37°C and absorbance at 240 nm is measured.

b. Incubation Assay--Each sample (0.1 ml) is mixed with 0.9 ml of 0.018 M H_2O_2 in 0.01 M phosphate buffer, pH 7.0, in 1.5-ml plastic tubes and these are placed in a 37°C water bath for 1 hr. The A_{240} of samples and catalase standards are measured and the activities of samples read from a standard curve produced with known catalase samples.

4. Diaphorase (24)

a. Kinetic Assay

Reagents:

<A> NADH: 4.0 mM in sodium phosphate buffer, 0.03 M, pH 8.0

<B> Tris acetate: 0.2 M, pH 8.0

<C> INT: 4 mg/ml in water

Procedure:

1) Pipette into a 1.0-ml cuvette 0.1 ml Reagent <A>, 0.88 ml Reagent <B> and 0.02 ml of Reagent <C>.
2) Place this mixture into a spectrophotometer set at 490 nm and thermostatted to 30°C.
3) The absorption is read for 1-2 min to establish a blank rate.
4) At 0 time rapidly add the enzyme and mix. Follow the increase in absorbance for 3 min with a chart recorder.
5) Calculate the initial rate and report as A/min.

5. Lactate Dehydrogenase

a. Kinetic Assay (25)--Lactate dehydrogenase activity is measured by the rate of decrease in absorbance or fluorescence of the substrate NADH as it is oxidized to NAD^+. The reagent used consists of 4 mM NADH and 10 mM pyruvate in 30 mM phosphate buffer, pH 7.4. A 0.1-ml sample is added to 0.9 ml reagent in a 1-ml cuvette in a 37°C sample chamber and the

Δ A was measured on a chart recorder.

b. Incubation Assay(26-27)--Smaller amounts of LDH are measured by an incubation-type assay which uses a dye as the final electron acceptor of the reaction. The reagent contained 0.14 mM NAD^+, 50 mM lactate, 40 μg/ml INT, and 1 U/ml diaphorase in 0.2 M Tris-acetate, pH 8.0. The 0.1-ml sample is added to 0.9 ml reagent in a 3-ml capped vial and 1 hr at 37°C. The INTF is extracted into 2 ml 1.5:1 tetrachloroethylene:acetone and quantitated at 490 nm in a spectrophotometer. The incubation time is varied to measure different ranges of enzymatic activity.

c. Enzyme Cycling Assay (20,28,29)--Extremely small amounts (1μU - 1 mU) of LDH are detected using enzymatic cycling to quantitate the NAD^+ produced by the reaction when pyruvate and NADH are substrates. The reagent contains 30 mM pyruvate and 10 mM NADH in 30 mM phosphate buffer pH 7.4. LDH reagent (10 μl) was mixed with 20-μl samples in 1.5-ml microfuge tubes and incubated 1 hr in a 37°C water bath. The reaction is stopped and excess NADH destroyed by adding 10 μl 1.0 N HCl. After 10 min the samples are neutralized with 10 μl 1.0 N NaOH. The enzymatic cycling procedure is used to amplify and quantitate the NAD^+ produced by LDH activity.

6. Microtox (Bacterial Luminescence) (29,30)

a. Preparation

Test samples are adjusted to the required NaCl concentration (2%) with 22% NaCl by mixing 10 parts sample with 1 part of the salt solution.

Several dilutions of the samples are normally tested to assure a reliable estimation of toxicity. The concentrations of sample used ranges from 0% (control) to 45%. Temperature equilibration is essential for all reagents.

The Microtox reagent is activated by reconstituting the freeze-dried cells with 1 ml of 3°C reconstitution solution. The reconstitution solution is added rapidly by pouring it into the vial containing the freeze-dried bacteria. The suspended bacteria are transferred back to the test tube originally containing the solution and the contents mixed by flicking. The reconstituted cells are stored at 3°C until needed. For assay the

suspended bacteria are dispensed (30 µl) into tubes containing 1.5 ml of 2% NaCl and kept in a 15°C incubator.

b. Measurement

1) Transfer 100 µliter of diluted Microtox reagent to each of six cuvettes in the Pico-Lite sample chamber.

With the cuvette chamber in UP position zero the recorder pen. Lower the cuvette chamber and measure and record the light output. The bacteria will display a characteristic light output pattern during their equilibration period. The normal pattern of light output is characteristically an increase in light intensity, a short period of constant light output, and then a decrease. The last phase of decreasing light output is usually characterized by an initial rapid light loss followed by a longer period of slower light loss.

This final phase of minimum light loss is the phase used in testing. Normally a 15- 20 min period of equilibration is required before the cells have reached the testing phase.

2) Scan cuvettes 1-6 to obtain relative light level of each. Only a few seconds are required. By repeating the scan cycle 3 times, a base line output will be recorded for each cuvette, represented by the peak height points on the chart recorder.

3) Add 100 µl of each solution to the appropriate cuvette, mix quickly and then cycle the cuvette positions to measure the light output as described previously. The cycling is continued for at least 5 min. The chart recorder can be used as the timing device since it operates at a known speed.

4) Data reduction--If proper sample concentrations are used, an approximate EC_{50} (5 min, 15°C) (concentration of toxicant or effluent causing 50% reduction in light after 5 min at 15°C) can be determined by conventional bioassay data reduction procedures such as log-linear plotting of concentration vs. per cent of light loss. The light loss is obtained using a blank correction, normalized with control

cuvettes, and expressed as per cent. When the sample is not very toxic, no EC_{50} can be obtained. In such cases the light loss after 2 min and/or 4 min is used (LL_2 & LL_4, respectively).

G. Lipopolysaccharide

1. Limulus Amebocyte Lysate Assay (39,40)

Reagents:

<A> Artificial Sea Water

Weigh out:

$NaCl$, 27.48 g
$MgCl_2$, 5.10 g
$CaCl_2$, 1.14 g
KCl, 0.74 g

Place these salts in treated 1 liter bottles (heated to 180° C overnight) and bake at 180° C overnight. Add 1 liter of pyrogen-free water and mix thoroughly. When salts are dissolved add 0.01 mole imidazole (0.68 g) using a treated (baked) spatula and weighing pan. Adjust the pH to 7.6 with pyrogen-free NaOH or HCl.

<B> Reconstitution of lyophilized LAL: (40)

1) Put 5-ml ampule sterile water on ice to cool. Sterilize the outside of the ampule with 70% ethanol and break open.
2) Using a 10-ml sterile syringe with 18-gauge needle, remove 10 ml water and add to lyophilized salt solution. Cover with the clean side of Parafilm, shake, and put on ice when dissolved.
3) Remove the sterile salt solution with a sterile pipette and add to lyophilized LAL. Cover with Parafilm; let set on ice for 20-30 min. If the protein is not dissolved, invert the bottle slowly.
4) Centrifuge in a refrigerated centrifuge in Corex tubes at 3000-4000 rpm for 10 min. Decant the supernatant solution from the pellet if one is present. Keep on ice during day while working. May be frozen one time for later use.

<C>. LPS Standards:

Prepare standards by dilution with pyrogen-free water to give final concentration of endotoxin of 100 ng/ml.

Procedure

1) Add 0.2 ml LAL to 1-ml sample in 10 x 75 mm pyrogen-free tube. Vortex gently.
2) Incubate for 1 hr in 37° C water bath. The

temperature should not vary more than ± 0.5° C.

3) Stagger sample preparation so that the absorption may be read shortly (5 min) after the end of the incubation.
4) Vortex gently; read absorption at 360 nm.
5) Plot curve of absorption versus standard LPS (50-1000 fg).

SECTION 6

RESULTS AND DISCUSSION

A. Protein Determinations

There are many procedures and variations thereof for the estimation of protein concentration. Each procedure is based on some property of the protein which is not universal and certainly is not manifested with the same frequency in the various proteins. Therefore, the determinations are at best estimates and are based on comparisons to a specific standard. Bovine serum albumin is the standard most often used.

Because the Lowry method (2) is superior to the standard biuret (45, 46) and the micro-Kjeldahl method (47), it was the starting point in our study of protein determination methods.

1. Lowry Protein Determination

Figure 2 shows the standard curve that we obtained using the Lowry procedure. The Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall (2) has been reviewed in detail by Peterson (48).

2. Dye-Binding Protein Determinations

The binding of certain dyes to proteins can be used as a protein estimation. Coomassie Brilliant Blue G-250 was used by Bradford (49) to establish a protein estimation with a sensitivity of about 10 μg of protein. McKnight (3) used glass fiber filters and accurately determined 0.1 μg of protein. Another variation of the procedure by Sedmark and Grossberg (50) was capable of detecting less than 1 μg. The variation in color yields from different proteins was noted by Pierce and Suelter (51) and by Van Kley and Hale (52). When the Bradford method was applied to crude cell and tissue extracts, there was a fairly consistent color yield (Chiappelli, Vasil, and Haggerty, 53). Figure 3 shows the standard curve that we obtained using Coomassie Blue.

Greif (54) used the anionic dye bromosulfalein to estimate protein concentrations in 1950. This assay has been improved by Nayyar and Glick (55) and by Bonting and Jones (56) to increase

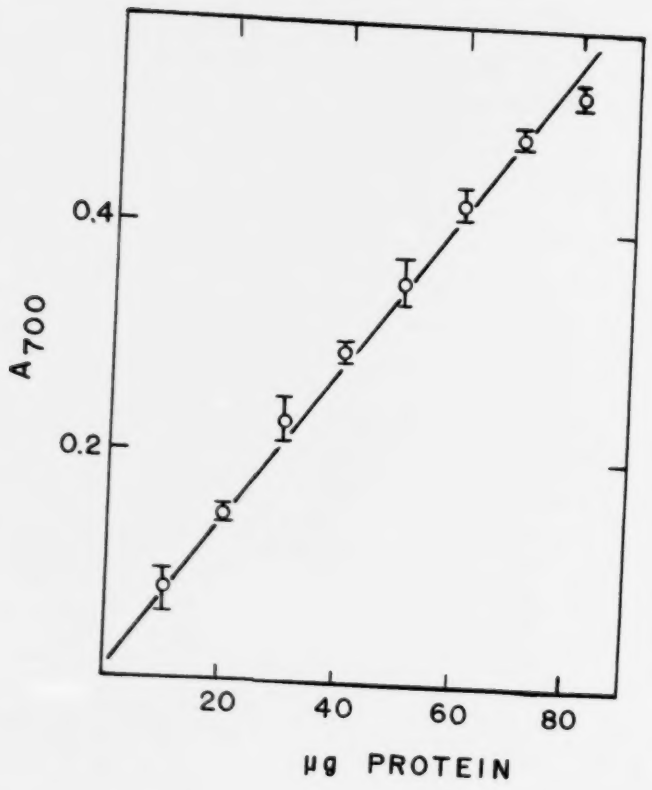


Figure 2. Standard curve for Lowry protein determination. The absorbance measured at 700 nm is plotted against the amount of bovine serum albumin. The procedure is detailed in Section 5.

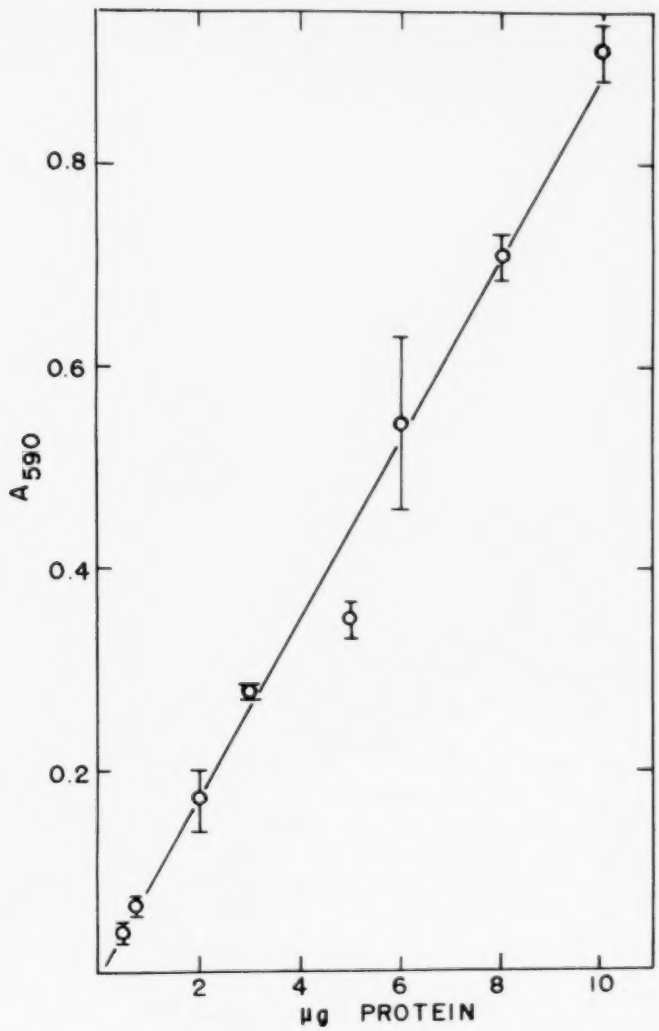


Figure 3. Standard curve for Coomassie blue protein determination. The absorbance measured at 590 nm is plotted against the amount of bovine serum albumin. The procedure is given in Section 5.

the sensitivity to 1 μg of protein. The procedure was modified by McGuire, Taylor, and Greene (4) for use over a range of 0.5 to 100 μg of protein. Our standard curve using this procedure is shown in Figure 4. Table 3 shows the variation in color produced in the protein assay when bromosulfalein is bound to different proteins.

Bromophenol blue can also be used (Flores, 57) but this assay is less sensitive — 10 μg of protein.

3. Radioisotopic Protein Determination

Schultz and Wassarman (58) used (^{3}H)dansylchloride to react with amino groups. When we applied the method, we found that the assay was linear over the range of 0.08 to 2.5 μg of protein, but reproducibility was poor (30% error). The reaction must be done in the dark, and the reaction conditions influence the extent of reaction. Burzynski (59) reported greater sensitivity when the reaction is done in capillary tubes.

The small increase in sensitivity achieved by these procedures was not significant when balanced against the difficulty of the procedure and the complexity of the equipment required.

4. Fluorimetric Protein Determinations

Two major reagents, fluorescamine and o-phthalaldehyde, have been used for the fluorimetric determination of protein. Bohlen, Stein, Dairman, and Udenfriend (60) developed a procedure using fluorescamine which assays 10-50 ng of protein (another range of sensitivity, 500 ng-50 μg, was also reported). Roth (6) described conditions for the fluorimetric assay of amino acids in the nanomole range using o-phthalaldehyde.

An assay for protein in the range of 0.1-50 μg/ml was developed by Weidekamm, Wallach, and Fluckinger (62). Benson and Hare (63) reported the o-phthalaldehyde assay to be 5-10 times as sensitive as that using fluorescamine.

The sensitivity of protein determination was increased by Butcher and Lowry (64) through hydrolysis of the protein to the component amino acids. They measured as little as 3 ng with o-phthalaldehyde, and by the use of enzymatic cycling to determine glutamate they were able to detect 1 ng of protein.

Figure 5 shows the standard curve that we obtained without hydrolysis while Figure 6 shows the standard curve when the protein was hydrolyzed. We found that 6 ng of bovine serum albumin was as little protein as we could detect. The main problem of the method is the background of protein that is present even on new test tubes. The test tubes were subjected to 3 cycles of acid hydrolysis using 6 N HCl at 110° C for 5 hr followed by extensive washing with water. Without using acid-treated test tubes and pipette tips, the detection limit varied between 30 and 100 ng.

Table 4 compares the sensitivities of the various protein

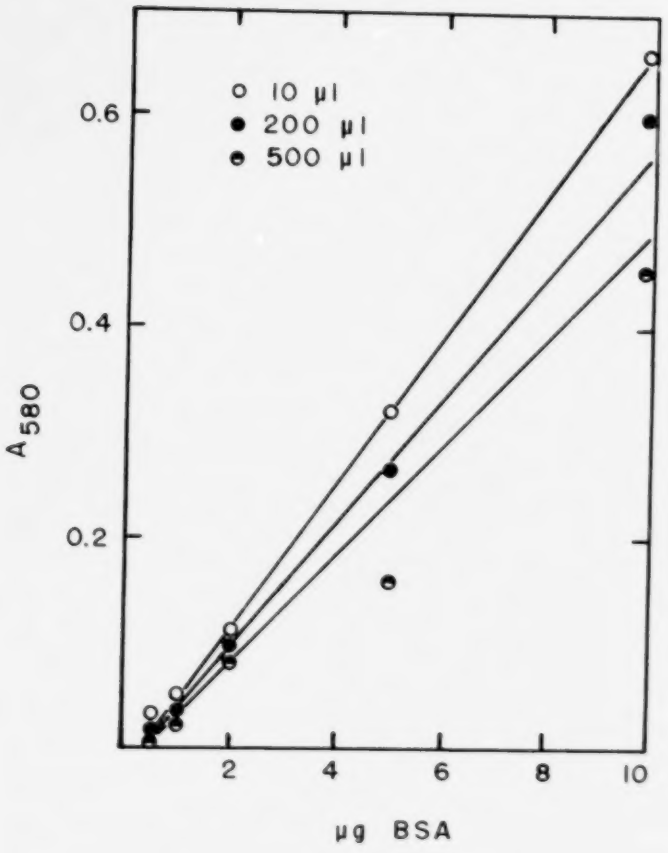


Figure 4. Standard curve for bromosulfalein protein determination. Effect of the volume of protein sample on the absorbance at 580 nm was determined. Bovine serum albumin was the standard protein and the determination was conducted as described in Section 5.

TABLE 3. ABSORBANCE AT 580 NM PRODUCED BY INTERACTION OF BROMOSULFALEIN WITH 5 μG OF VARIOUS PROTEINS

Protein or compound	A_{580}
Polyglutamate	0.010
Bovine serum albumin	0.267
Pepsin	0.044
Trypsin	0.004
Casein	0.146
Ovalbumin	0.213
Polylysine	0.709

TABLE 4. SENSITIVITIES OF VARIOUS PROTEIN DETERMINATION METHODS

Method	Lowest Amount of BSA Detectable
Lowry	10 μg
Coomassie blue	0.5 μg
Bromosulfalein	1 μg
o-Phthalaldehyde (without hydrolysis)	30 ng
o-Phthalaldehyde (with hydrolysis)	6 ng

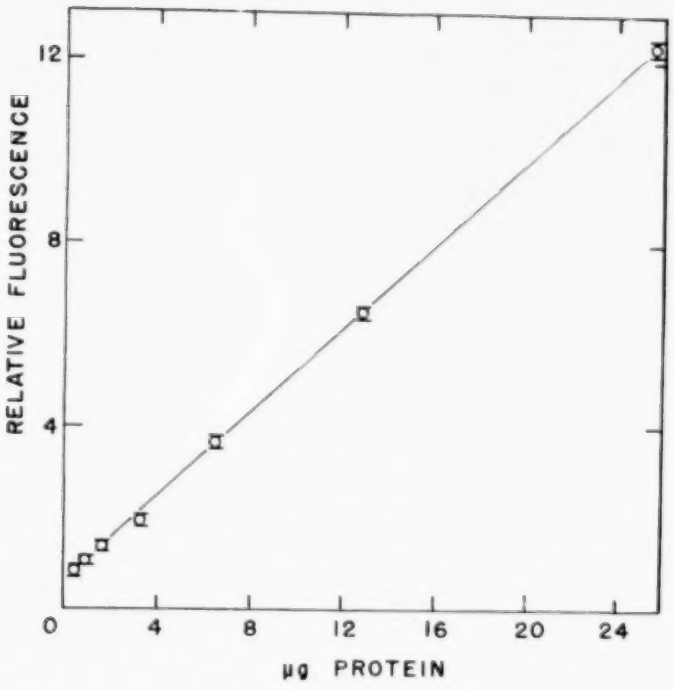


Figure 5. o-Phthalaldehyde protein determination without hydrolysis. The relative fluorescence is plotted against the amount of bovine serum albumin. The procedure is described in Section 5.

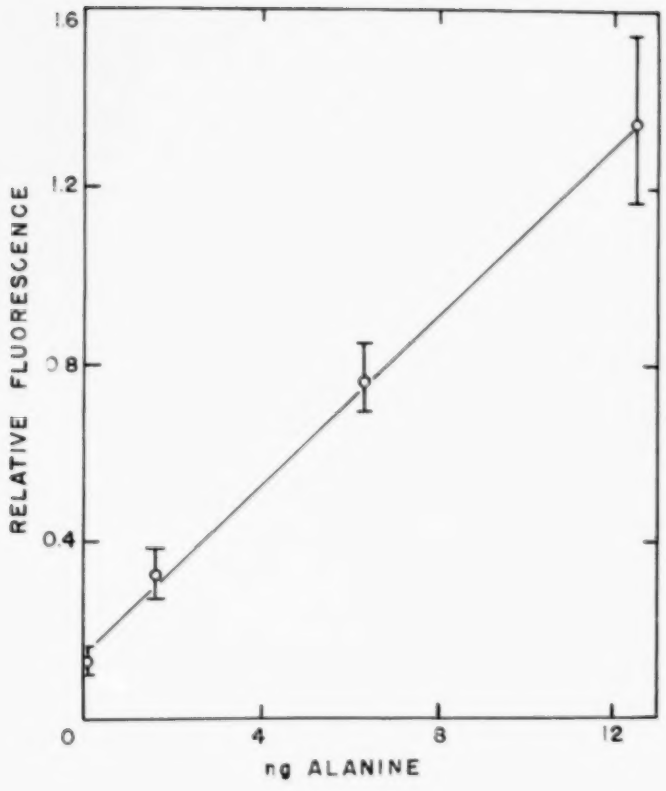


Figure 6. o-Phthalaldehyde protein determination with hydrolysis. The standard curve of relative fluorescence versus alanine concentration is shown. The alanine was subjected to the acid hydrolysis procedure as described in Section 5.

estimation techniques that we used.

5. Other Protein Determination Methods

Goldberg (65) described an ultrasensitive assay that gave a linear response between 10 and 20 ng of protein; this was based on copper reaction with the protein, the copper present in the complex being determined with phenol and chloramine-T. A microassay based on the fluorimetric determination of a protein dansylated in the presence of cycloheptaamylose developed by Kinoshita, Iinuma and Tsuji (66) detected 50-400 ng of protein.

Viets, Deen, Troy, and Brenner (67) report that the sensitivities of measuring total protein in nanoliter samples were similar for fluorescamine and o-phthalaldehyde. They found that the o-phthalaldehyde method was simpler and more reproducible. Alkaline hydrolysis of the protein of dental plaque followed by o-phthalaldehyde amino determination gave a sensitivity of about 10 ng (68).

Because of lower blank values, Castell, Cervera, and Marca (69) recommend use of fluorescamine for low concentrations of protein even though the fluorescence produced by reaction with o-phthalaldehyde is greater.

Microfluorimetric procedures using picoliter samples (Mroz and Lechene, 79), and the use of microliter samples in disposable reaction chambers (Leaback and Creme, 71) have been described.

B. DNA Determinations

1. Diphenylamine DNA Determination

The most frequently used colorimetric procedure for DNA determination is based on the reaction of diphenylamine with the deoxyribose residues. Table 5 shows the evolution of this assay with a 50-fold increase in sensitivity achieved through the various modifications. A standard curve for the diphenylamine assay is shown in Figure 7.

2. Diaminobenzoate DNA Determinations

The diaminobenzoic acid assays offer advantages over diphenylamine, e.g., shorter time for color development, use of a less hazardous reagent, and suitability of the same reagent for both a spectrophotometric and a fluorimetric assay (8). The spectrophotometric standard curve is shown in Figure 8 and the fluorimetric curve in Figure 9.

3. Ethidium Bromide DNA Determination

Ethidium bromide interacts strongly with double-stranded DNA to form a complex having enhanced fluorescence. Figure 10 shows a standard curve produced with this reagent.

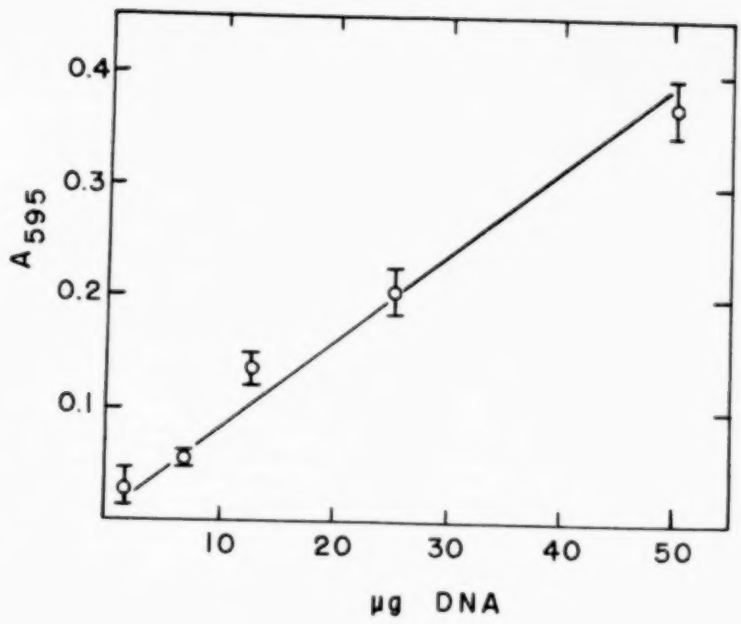


Figure 7. Standard curve for diphenylamine DNA determination. The absorbance at 595 nm is plotted against the amount of salmon sperm DNA. The procedure is described in Section 5.

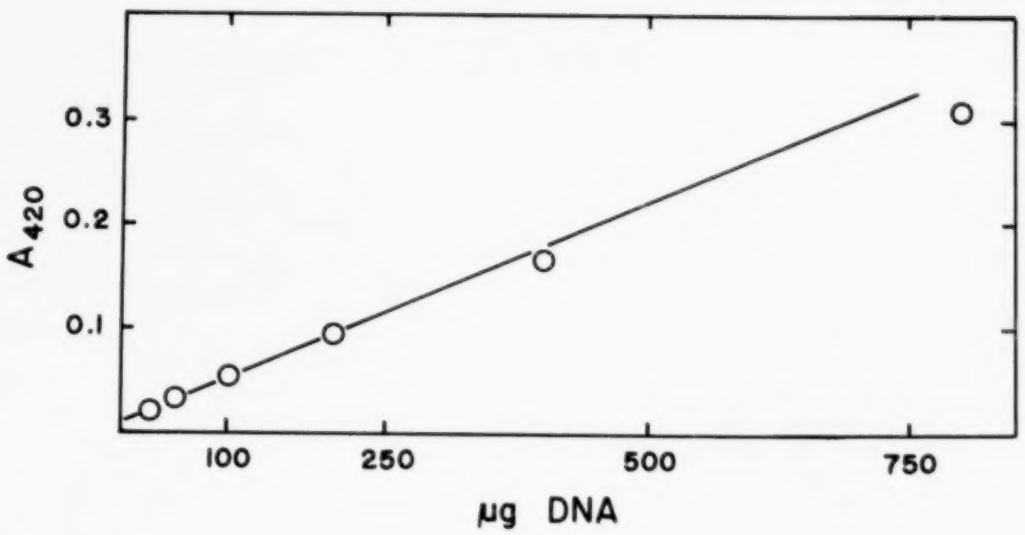


Figure 8. Diaminobenzoate spectrophotometric DNA determination. The absorbance at 420 nm is plotted against the amount of salmon sperm DNA. The procedure is described in Section 5.

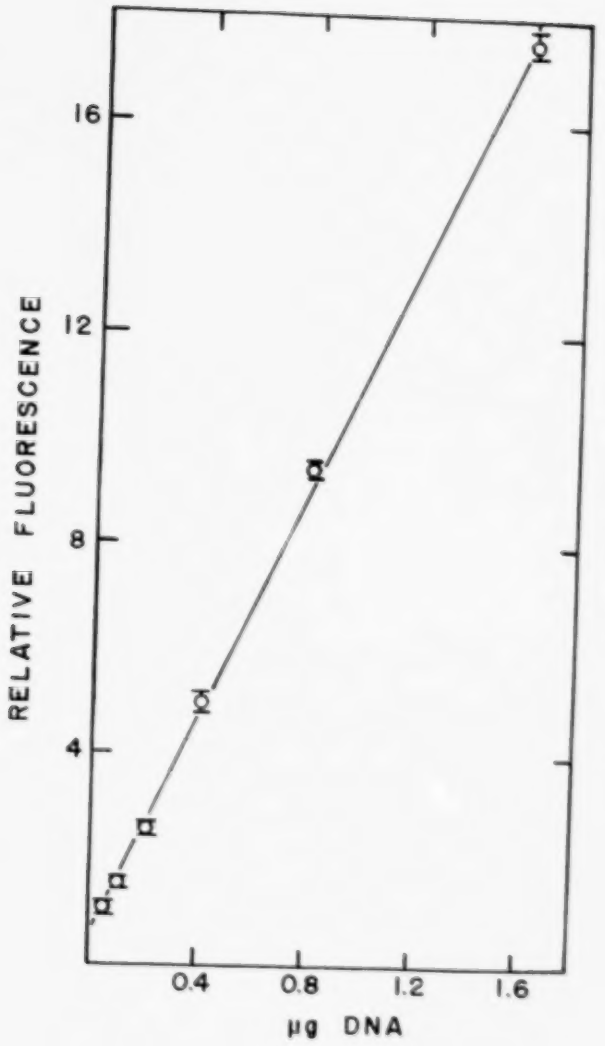


Figure 9. Diaminobenzoate fluorimetric DNA determination. The relative fluorescence is plotted against the amount of DNA as described in Section 5.

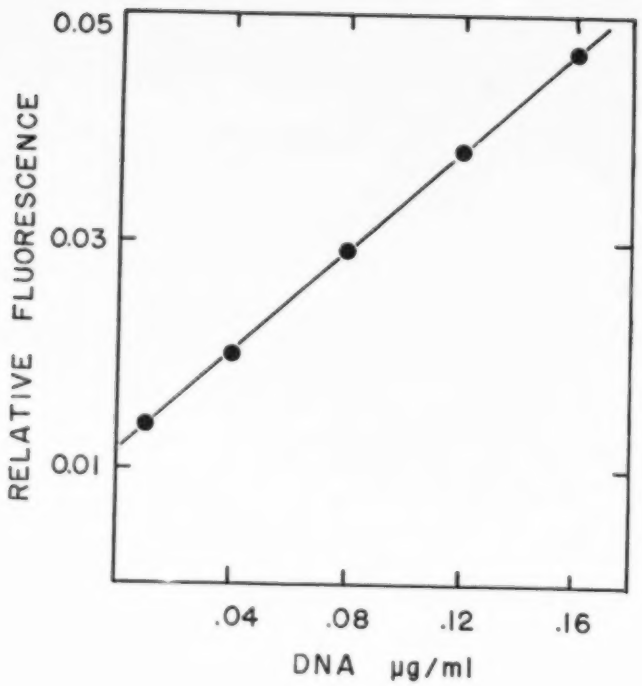


Figure 10. Ethidium bromide fluorimetric DNA determination. The relative fluorescence produced upon ethidium bromide binding to salmon sperm DNA was determined as described in Section 5.

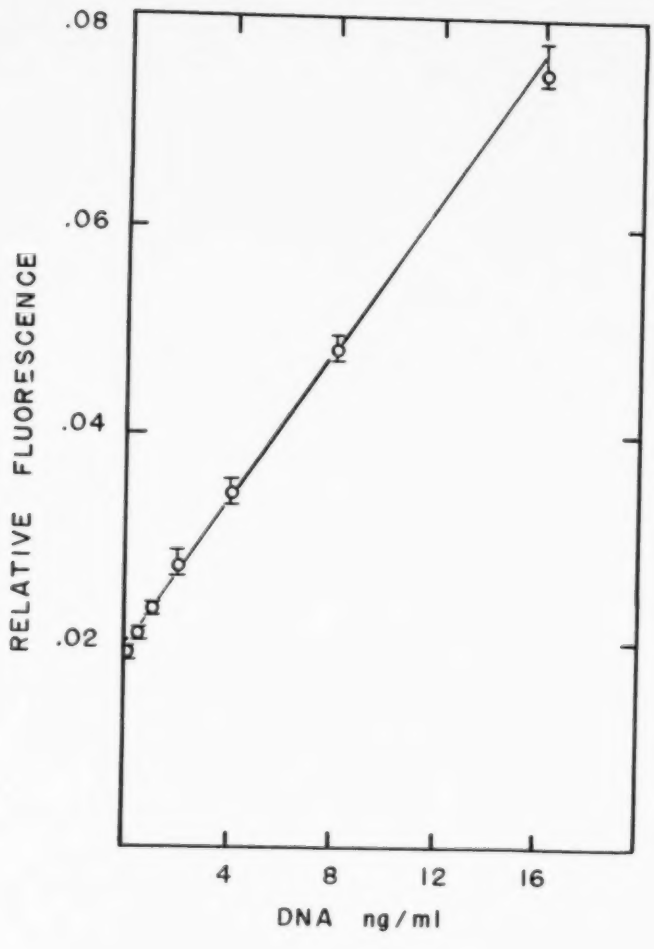


Figure 11. DAPI fluorimetric DNA determination. The procedure is detailed in Section 5 for the measurement of the relative fluorescence produced upon binding of DAPI to DNA. The relative fluorescence is plotted against the DNA concentration.

TABLE 5. MODIFICATIONS OF THE DIPHENYLAMINE METHOD FOR DNA DETERMINATION

Author	Modification	Sensitivity (μg)
Dische (72)	Original, 10 min, 100°C	50
Burton (73)	Acetaldehyde, 17 hr, 30°C	10
Giles and Myers (74)	$-H_2SO_4$, different concentrations of acetaldehyde added	1.5
Richards (75)	Paraldehyde	1

TABLE 6. COMPARATIVE SENSITIVITIES OF METHODS FOR DETERMINING DNA

Method	Sensitivity (ng/ml)	E. coli (minimum #)
Diphenylamine	1000	6×10^7
Diaminobenzoic acid	30	2×10^6
Ethidium bromide	10	6×10^5
DAPI	0.5	3×10^4

TABLE 7. COMPARATIVE SENSITIVITIES OF RNA DETERMINATION METHODS

Method	Range of Response	E. coli (# required)
Orcinol (colorimetric)	1-50 μg	1×10^8
Ethidium bromide (fluorimetric)	5-20 ng	1×10^6

4. DAPI DNA Determination

DAPI (4 ',6-diamidino-2-phenylindole) also reacts with DNA to form a fluorescent complex. This method is the most sensitive assay for DNA as can be seen from Figure 11. Labarca and Paigen (76) suggest that bisbenzimidole (Hoechst 33258) has the advantage over DAPI in that the blank is lower, and that the reaction is linear over a broader range of DNA concentrations. Their stated sensitivity is 10 ng.

A comparison of these methods for DNA assay is shown in Table 6 with the number of E. coli cells required to give that amount of DNA.

5. Other Methods of DNA Determination

Several other methods have been used for DNA determination. Martin, Donohue, and Finch (77) used p-nitrophenylhydrazine and found a sensitivity of 10 ug; Gold and Shochat (78) found a sensitivity of 0.5 ug with thiobarbituric acid; Cesarone, Bolognesi, and Santi (79), using Hoechst 33258 observed a sensitivity of 0.25 ug (79); Labarca and Paigen (76) also used bisbenzimidazole (Hoechst 33258) and detected as little as 10 ng; Brunk, Jones, and James (80) used both DAPI and Hoechst 33258 to measure DNA in the nanogram range; and Reis (81) applied a microwell-microdensitometer method with ethidium bromide, ultraviolet irradiation and photography to achieve a sensitivity of 10 ng.

When a dimeric derivative of ethidium bromide was used, Markovits, Roques, and LePecq (82) found a 100-fold increase in the sensitivity of the fluorimetric DNA determination, so that they were able to estimate DNA concentrations as low as 1 ng/ml.

C. RNA Determinations

1. Orcinol RNA Determination

There are two variations of the orcinol reaction with pentoses; $FeCl_3$ (83) is the catalyst in one and $CuCl_2$ (84) in the other. Several modifications have been made, but will not be discussed here (see Dische (85)). The modification that we used was that of Ceriotti (15). The standard curve is shown in Figure 12. A recent modification by Almog and Shirey (86) improves the specificity of the reaction for RNA. The standard curve they observed was linear between 10 and 200 ug of RNA; however, the procedure takes longer because of a 24-hr incubation with H_2SO_4.

2. Ethidium Bromide RNA Determination

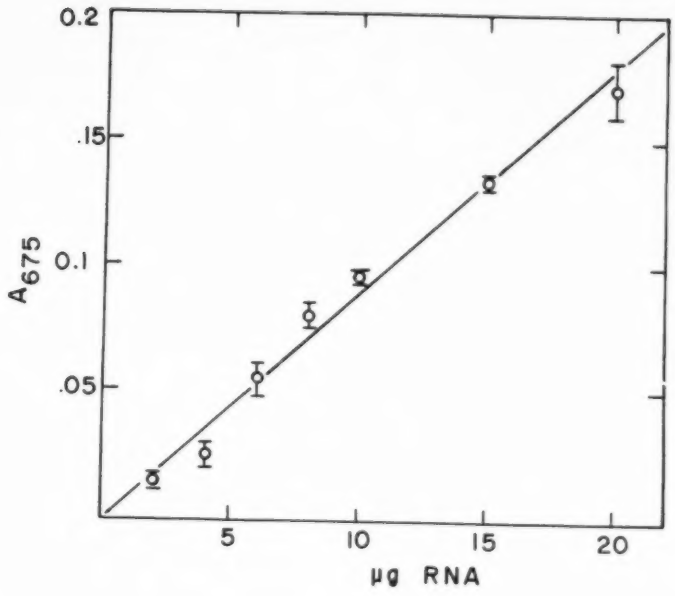


Figure 12. Standard curve for orcinol RNA determination. The absorbance produced through reactions of orcinol and RNA was determined at 675 nm. The procedure is detailed in Section 5. The absorbance is plotted against the RNA concentration.

Ethidium bromide has been used for the determination of both DNA and RNA (10). The application of ethidium bromide to DNA and RNA determination in mammary gland homogenates has been reported by Beers and Wittliff (87). Pea seed samples were used by El-Hamalawi, Thompson and Barker (13) and improvements in the method were reported by Karsten and Wollenberger (12).

The standard curve for RNA determination using ethidium bromide is shown in Figure 13. Table 7 compares the colorimetric and fluorimetric procedure.

D. Organic Phosphate Determination

1. Phosphate Determination

There are many phosphate determinations, both inorganic and organic, because of the importance of both kinds in biochemical reactions. Many of these methods are modifications of the Fiske and SubbaRow procedure (88). We used the Lazarus and Chou (89) modification; the standard curve is shown in Figure 14. The procedure of Chen, Toribara and Warner (90) has the same sensitivity. Extraction of the reduced molybdoantimonylphosphoric acid with acetophenone-chloroform (Going, Wenzel and Thompson, 16) removes the interference by silicates. The standard curve for this determination is shown in Figure 15.

2. Organic Phosphate Determination

The method has been applied to ATP with the results shown in Figure 16 and to *E. coli* cells where a content of 6 fg were obtained per cell; this agrees with the known value.

A microfluorimetric method in which phosphate is converted to hexadimolybdatophosphate, which by reaction with thiamine produces thiochrome, has been described by Brunette, Vigneault, and Danan (91).

E. Coenzyme Determinations

All living organisms obtain energy from their environment (by photosynthesis or substrate oxidation) and use that energy for growth and reproduction. Respiratory cofactors, which function in the transfer and storage of energy in the cell, include pyridine nucleotides (NAD^+/NADH and $NADP^+$/NADPH), adenosine triphosphate, flavin nucleotides (FMN/$FMNH_2$ and FAD/$FADH_2$) and iron porphyrins.

The approach for study of respiratory cofactors was similar to that for determination of enzymatic activities. Available assays were tested for sensitivity and improved where possible. The limits of detection were determined and bacterial cells were

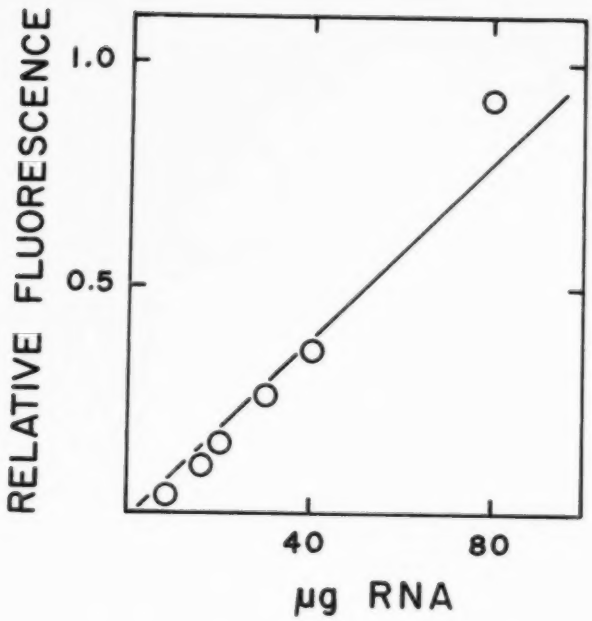


Figure 13. Standard curve for ethidium bromide RNA determination. The procedure described in Section 5 was used to measure the relative fluorescence when ethidium bromide reacts with RNA. The relative fluoresence is plotted against the RNA concentration.

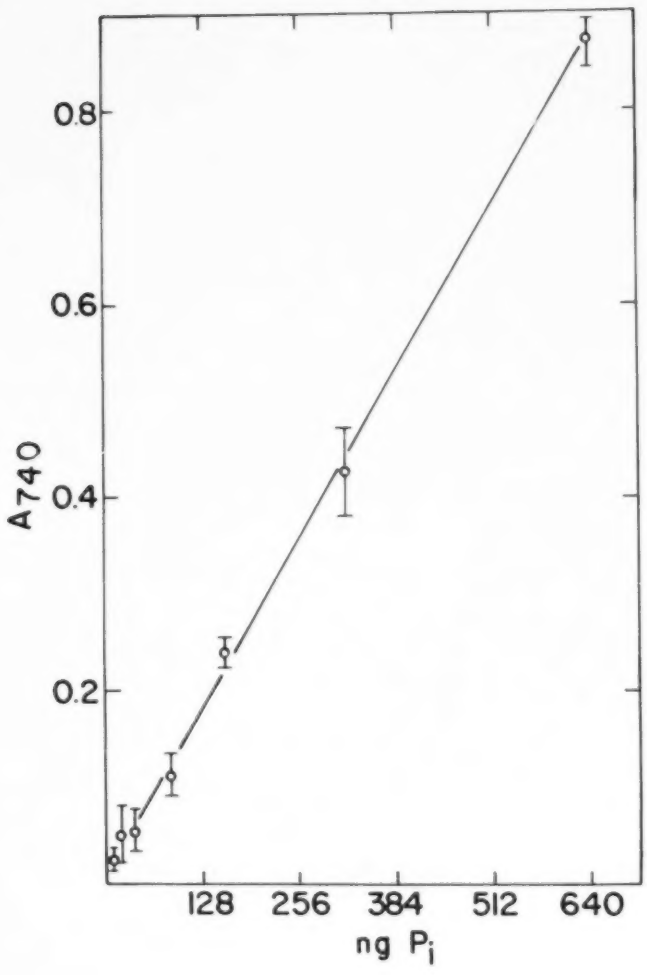


Figure 14. Standard curve for phosphate determination. Phosphate was determined as described in Section 5. The absorbance at 740 nm is plotted against the amount of inorganic phosphate.

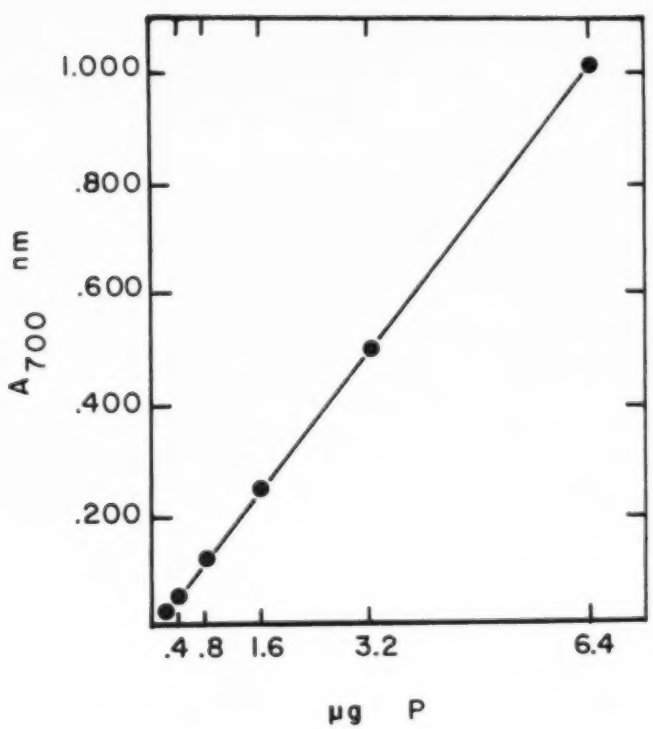


Figure 15. Standard curve for phosphate determination with extraction. When an extraction step performed as described in Section 5 is added to the phosphate determination procedure, an increased sensitivity results.

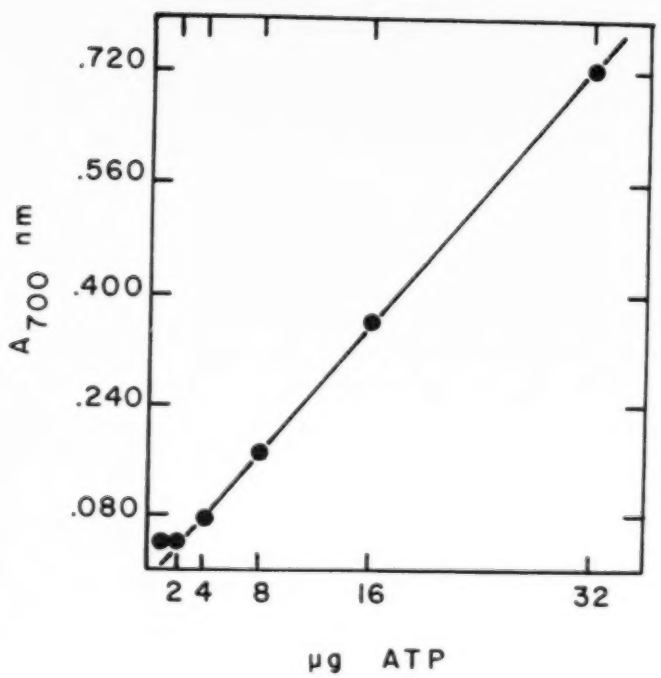


Figure 16. Determination of ATP by phosphate analysis. The amount of phosphate present in various amounts of ATP was determined by the extraction procedure described in Section 5. The absorbance at 740 nm is plotted against the amount of ATP.

assayed, using extraction techniques where appropriate, for each respiratory cofactor.

1. Pyridine Nucleotides

Pyridine nucleotides are quantitated by the absorbance or fluorescence of the reduced pyridine nucleotides NADH and NADPH or by luminescence produced by bacterial luciferase. The response of $NAD(P)^+$ /NAD(P)H can be amplified by enzymatic cycling to improve the sensitivity of detection of these nucleotides.

a. Direct Measurement of NADH and NADPH

i. Spectrometry and Fluorometry — NADH was quantitated spectrophotometrically (340 nm) and fluorometrically (excitation 340 nm, emission 455 nm). The spectrophotometric limit of detection was 0.8 nmol and the fluorometric limit of detection 0.071 nmol NADH in a 1-ml cuvette using water or buffer as a blank. The responses for NADPH are the same as for NADH (92) and were not tested.

ii. Luminescence Using Bacterial Luciferase — The coupled enzymes NADH: FMN oxidoreductase-bacterial luciferase were used to measure NADH (Figure 17). Two crude enzyme preparations were used in the determinations but they had inherent light production which made the method too insensitive for application to bacterial samples. Before further work is undertaken, the enzymes involved will have to be purified and prepared in a commercially useful form as is the case with firefly luciferase.

b. Enzymatic Cycling of Pyridine Nucleotides

The enzymatic cycling procedure of Lowry and Passonneau (20) was used to amplify the signal produced by pyridine nucleotides in a sample. Since it is possible to couple many enzymatic activities and metabolites to pyridine nucleotides, a number of other bioindicators can be detected through determinations of pyridine nucleotides by enzymatic cycling.

Calculation of Cycling Rate

The amplification obtained by enzymatic cycling is dependent upon the overall cycling rate, which is dependent upon the ratio of the two enzymes, glucose 6-phosphate dehydrogenase and glutamate dehydrogenase. The appropriate amounts of enzymes and the overall cycling rate were calculated by measurement of several parameters of the reaction (Figure 18). The ratio of oxidized/reduced pyridine nucleotide is given by the ratio of fluorescence intensities (C-E)/(C-A) from Figure 18 and the overall cycling rate calculated by using the amounts of substrates present and the time required for 90% oxidation of glucose 6-phosphate.

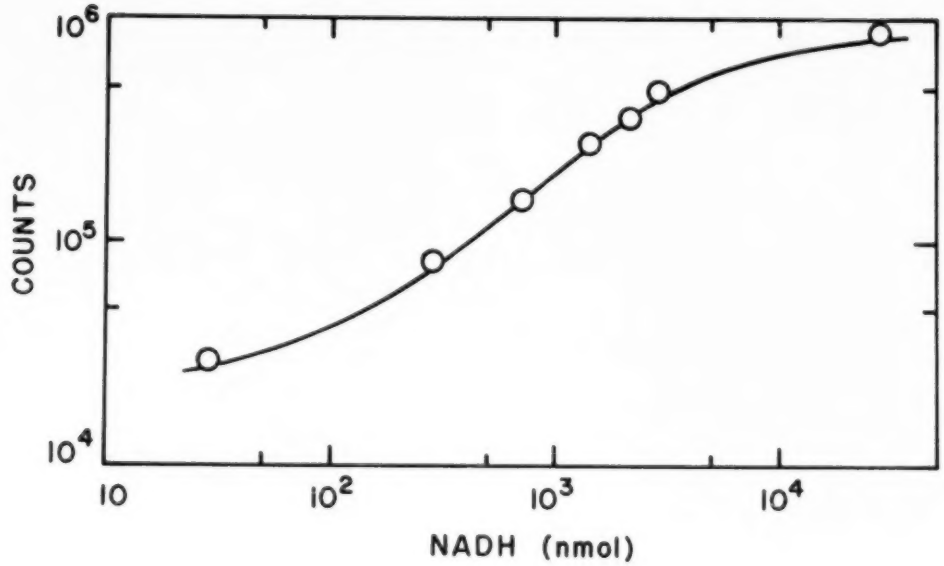


Figure 17. NADH measurement using bacterial luciferase. The luminescence produced by bacterial luciferase-NADH:FMN oxidoreductase in the presence of NADH was measured by integration of the light emission for 5 min after injection of 0.1 ml NADH sample into 0.4 ml bacterial luciferase reagent containing 9.7 nmol FMN and 10 µg dodecanal in 0.1 M phosphate buffer, pH 6.9. The amount of enzymes in the reagent was not determined.

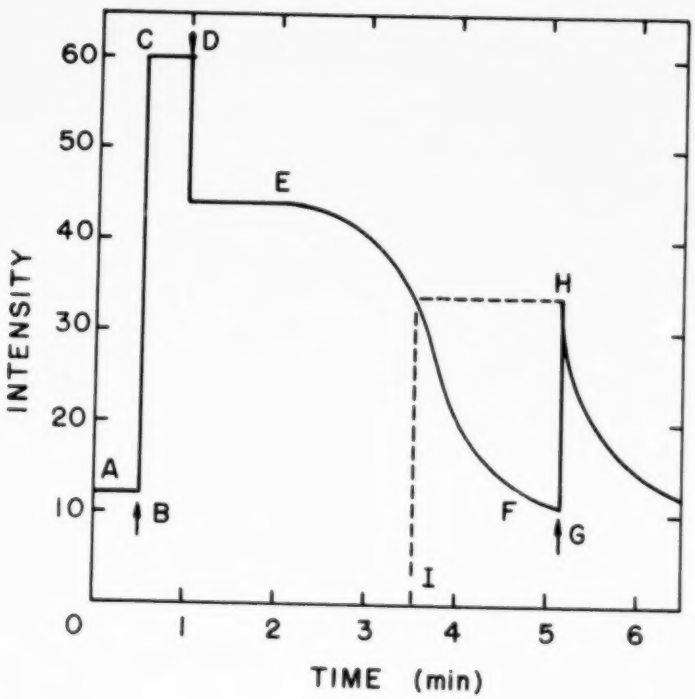


Figure 18. Measurements for calculation of cycling rate. Cycling reagent (1.0 ml) containing 10 nmol $NADP^+$ and 1 μmol glucose-6-phosphate but no enzymes was placed in a fluorometer (excitation 340 nm, 1-mm slit; emission 455 nm, 2-mm slit) with the sample chamber maintained at 37° C (A). Glucose-6-phosphate dehydrogenase (0.5 U) was added (B), reducing all the $NADP^+$ to NADPH (C). Glutamate dehydrogenase (0.72 U) was added (D) and the cycle started, producing an equilibrium concentration of NADPH (E). After the glucose-6-phosphate was exhausted (F), 0.1 μmol glucose-6-phosphate was added to the reaction mixture (G) to determine the NADPH concentration (H) and time (I) when 90% of the glucose-6-phosphate had been utilized. The time (I) was then used to calculate the overall cycling rate.

$$\text{Cycling Rate/hr} = \frac{\text{90\% Glucose 6-P (mol)}}{\text{NADP}^+ \text{ (mol)}} \times \frac{1}{\text{hr for 90\% G 6-P oxidation}}$$

The cycling rates of NAD^+ and $NADP^+$ differ considerably because of the specificities of the enzymes used for cycling. The cycling rate was 3600/hr for $NADP^+$ and 800/hr for NAD^+. This difference was more pronounced under actual assay conditions. The actual amplification of $NADP^+$ obtained by enzymatic cycling was 10,000/hr, determined by 6-phosphogluconate standards, while that for NAD was 800/hr. It is not clear what causes this difference but the maximum cycling rate of $NADP^+$ may occur at concentrations much lower than those used for the calculation of cycling rate.

c. Measurement of Pyridine Nucleotides

$NADP^+$ and NAD^+ were measured separately by the enzymatic cycling procedure (Figure 19). The incubation time was 1 hr for both pyridine nucleotides; because of the different cycling rates, $NADP^+$ measurement was more sensitive than NAD^+ measurement. The $NADP^+$ range was $5X\ 10^{-14}$ - $5X\ 10^{-12}$ mol and the NAD^+ range was $5X\ 10^{-12}$ - $5X\ 10^{-10}$ mol, with limit of detection for each being the low value of the range.

d. Measurement of Pyridine Nucleotides in *E. coli*

Pyridine nucleotides were extracted from *E. coli* into boiling Tricine buffer and measured by enzymatic cycling. The results were reported as $NADP^+$ since $NADP^+$ standards were used but the extracts actually contained a mixture of $NAD(P)^+$ and NAD(P)H. The extraction was tested using $NADP^+$ standards and a 96% recovery of $NADP^+$ was measured so no correction was made in the values determined for cell samples.

Two samples containing 5×10^8 and 5×10^7 cells were extracted with 5 ml boiling Tricine and 0.05-ml samples of the extracts were used in the cycling procedure. The amounts of $NADP^+$ measured in the cell samples were 1.4×10^{-12} mol/50 ul of the 5×10^8 cell extract and 1.7×10^{-13} mol/50 ul of the 5×10^7 cell extract. This may be expressed as 1.4×10^{-10} mol $NADP^+$ /5×10^8 cells and 1.7×10^{-11} mol $NADP^+$/ 5×10^7 cells. The limit of detection was 2.4×10^5 cells carried through extraction and cycling.

2. Flavin Mononucleotide

Measurement of flavin mononucleotide (FMN) was undertaken using bacterial luciferase with NADH and flavin reductase acting

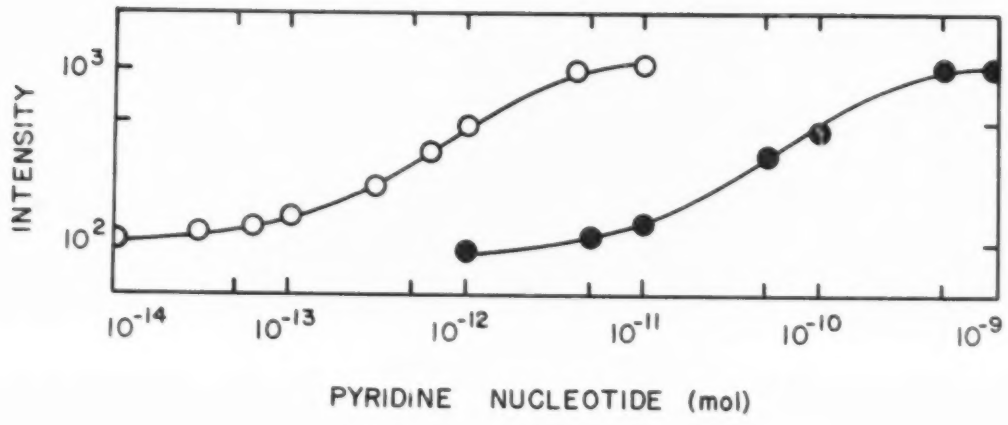


Figure 19. Measurement of $NADP^+$ and NAD^+. $NADP^+$ (O) and NAD^+ (●) were measured using the enzymatic cycling procedure. Samples (50 µl) were incubated one hour with 100 µl cycling reagent at 37° C. Fluorescence of NADPH produced by oxidation of the cycling product 6-phosphogluconate by 6-phosphogluconate dehydrogenase was measured (excitation 340 nm, 1-mm slit; emission 455 nm, 2-mm slit) and plotted against the amount of pyridine nucleotide present in the sample.

to generate the true reaction substrate, $FMNH_2$. A crude preparation of bacterial luciferase was used with an excess of NADH present so that light production was proportional to the amount of FMN in the sample. Aging the luciferase preparation to lower its blank luminescence also decreased its response to FMN and so the sensitivity of the assay was limited by the blank luminescence of the reagent. The limit of detection of the assay was 1 ng FMN and the range tested was 1-50 ng FMN (Figure 20). This was done using the procedure described in Section 5 with the JRB photometer.

With extra care and using the Pico-Lite photometer a greater sensitivity can be obtained as seen in Figure 21.

3. Iron Porphyrins

Iron porphyrins, that is porphyrin ring structures containing quadridentate-chelated iron atoms, act in the transfer of electrons from reduced cofactors (NADH and $FADH_2$) to molecular oxygen in aerobic organisms and as catalytic sites in enzymes such as catalase and horseradish peroxidase. Proteins containing either covalently or noncovalently bound iron porphyrins are sometimes called heme proteins.

Heme-containing proteins can be quantitated by their catalytic effect on the chemiluminescent oxidation of luminol under alkaline conditions. The major application of iron porphyrin determinations has been estimation of biomass or cell concentrations in environmental samples (34).

a. Light Emission During Luminol Oxidation

The intensity and duration of luminol chemiluminescence depends on the concentrations of reactants -- luminol, hydrogen peroxide, catalyst, and hydroxide ions (pH). Studies of reaction conditions have established the optimum reactant concentrations (34, 93), and these were used as a basis for applying the procedure to quantitation of iron porphyrins and biomass in this laboratory.

Luminescence measurements were made with the Pico-Lite photometer for peak height measurements. A hemoglobin standard curve is shown in Figure 22

b. Measurement in <u>E. coli</u>.

The limit of detection of bacteria by the luminol reaction has been reported as 10^4 /ml (34). Using peak height measurements in the Pico-Lite photometer 1×10^3 is the minimum number of cells detectable (see Figure 23).

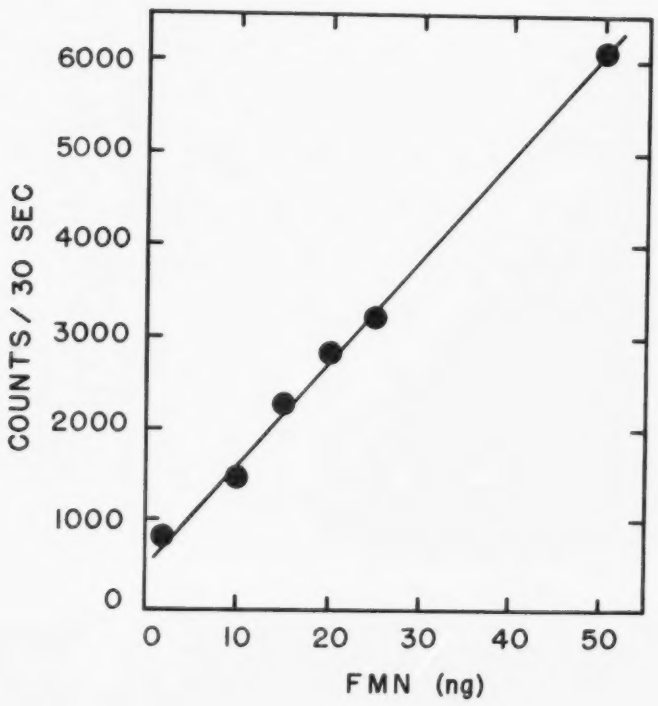


Figure 20. Measurement of FMN using bacterial luciferase. The luminescent reaction of bacterial luciferase was used to quantitate FMN as well as NADH. FMN samples (0.2 ml) were mixed with 0.3 ml bacterial luciferase reagent containing 0.2 mg luciferase-flavin reductase, 20 μg NADH, and 1.5 μg dodecanal in 0.4 M Tris-HCl buffer, pH 6.4. Samples were counted for 30 sec in the SAI Model 3000 Photometer using a 10-sec delay and sensitivity of 4.0.

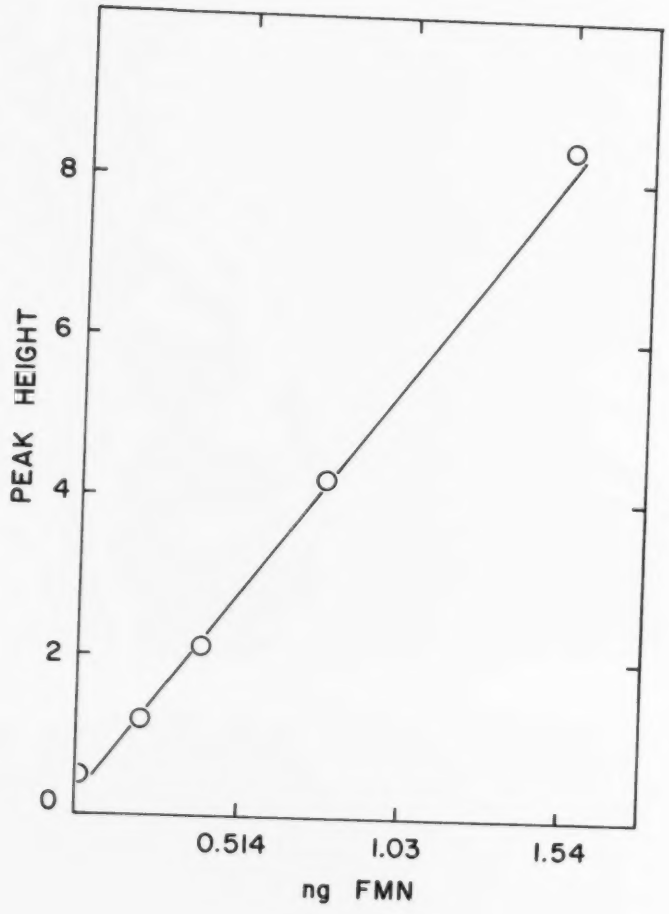


Figure 21. Measurement of FMN in the Pico-Lite using bacterial luciferase. The peak height light emission as read from a recorder trace is plotted against the FMN concentration. The assay was performed as described in Section 5.

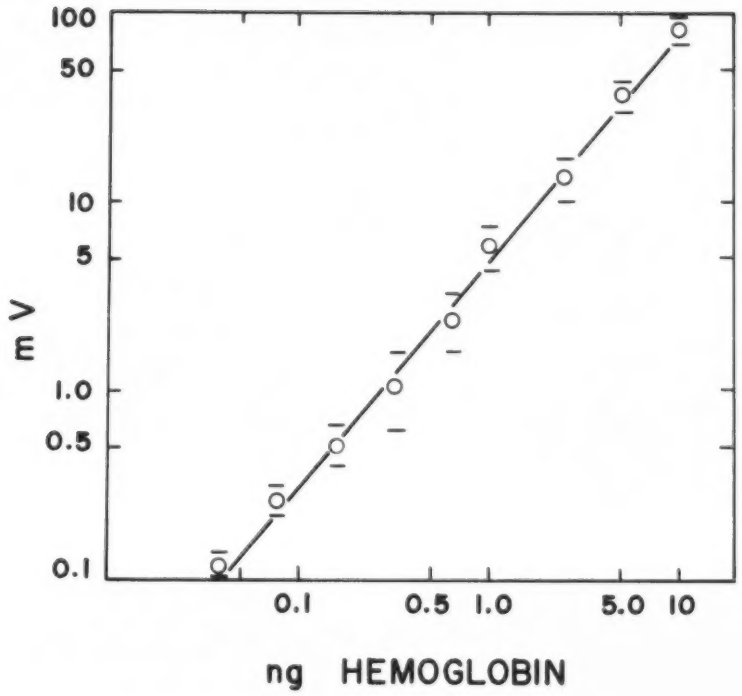


Figure 22. Hemoglobin standard curve using luminol. The logarithm of peak height voltage determined from a recorder trace is plotted against the logarithm of the amount of hemoglobin. The reaction was done as described in Section 5.

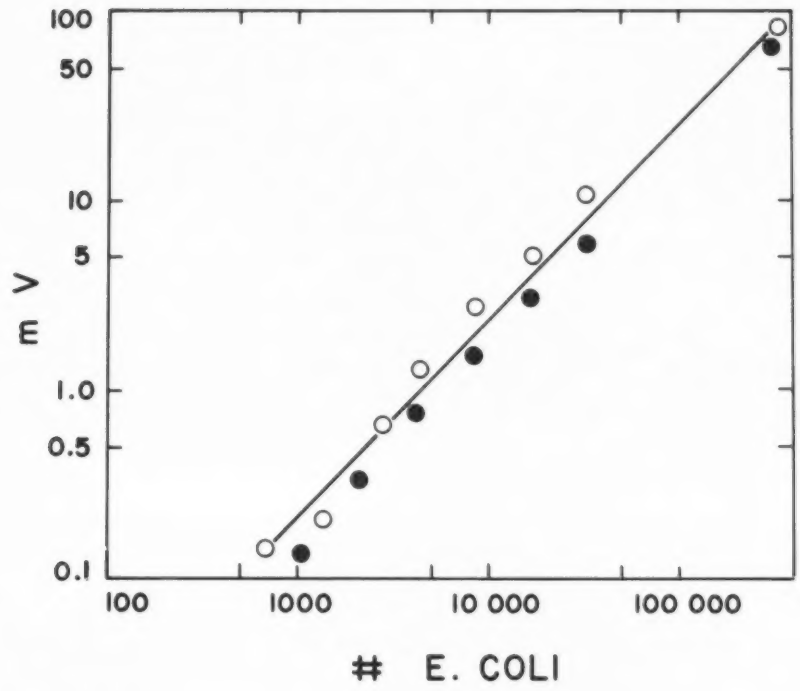


Figure 23. E. coli determination using luminol. Results from two separate experiments are shown. The logarithm of the peak height voltage read from a recorder trace is plotted against the logarithm of the number of E. coli cells. The cells were treated and the assay performed as described in Section 5.

F. Enzymes and Enzyme Systems Determinations

Introduction

Enzymatic activities are measured by product formation or substrate utilization. This may be done by kinetic assays where the rate of the enzymatic reaction is measured, or by determination of total product at the end of a timed incubation. The sensitivity of an enzyme is determined by that inherent in the analytical method used. The enzymes studied, lactate dehydrogenase, alkaline phosphatase, catalase, and adenylate kinase were measured by several assay methods. The sensitivity of each assay was determined so that an assay method with high sensitivity could be selected and used to determine the amount of enzyme in bacterial samples. E. coli was used as a model to test the selected enzyme assays for sensitivity and to determine cell numbers required to yield measurable enzymatic activity.

1. Adenylate Kinase

The ratio of adenine nucleotide concentrations in the cell is regulated by adenylate kinase (E. C. No. 2.7.4.3). Enzyme activity was determined by quantitation of ATP produced when ADP was used as substrate for the hexokinase- glucose 6-phosphate dehydrogenase coupled assay and by firefly luciferase reaction. The coupled enzyme assay was used for this study because the firefly luciferase preparations themselves had adenylate kinase activity which interfered with the determination of small amounts of enzyme.

a. Kinetic Assay.

Adenylate kinase was measured by following the rate of NADPH formation spectrophotometrically (Figure 24). The limit of detection was 3 ng and the range tested was 3-75 ng adenylate kinase. The contamination of commercial ADP with ATP is significant. For example, an ADP preparation from Sigma contained 1.3% ATP, and an older preparation from Pabst Laboratories, 2.4% ATP. Subsequent assays were performed with an ADP preparation that had been treated with hexokinase and glucose to remove ATP.

b. Incubation Assay.

A 1-hr incubation assay was used to determine whether the sensitivity of the kinetic assay of adenylate kinase could be improved. The same reagent was used but the incubation was at 37° C instead of 30° C. Adenylate kinase was measured by NADPH formation spectrophotometrically (Figure 25). The limit of detection was 0.1 ng and the range was 0.1-5.0 ng adenylate kinase. This represents a 30-fold increase in sensitivity over the kinetic assay.

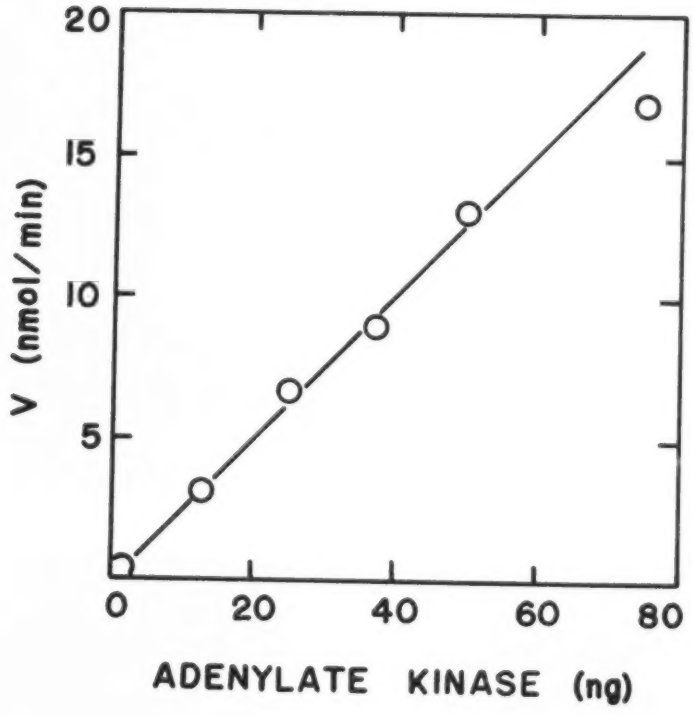


Figure 24. Kinetic assay of adenylate kinase. Adenylate kinase samples (0.1 ml) were mixed with 0.9 ml reagent in a 1.0-ml cuvette in a spectrophotometer thermostated to 30° C. The rate of NADPH formation measured at 340 nm using a calibrated chart recorder at 5 cm/min. The limit of detection was a Δ 0.005/min.

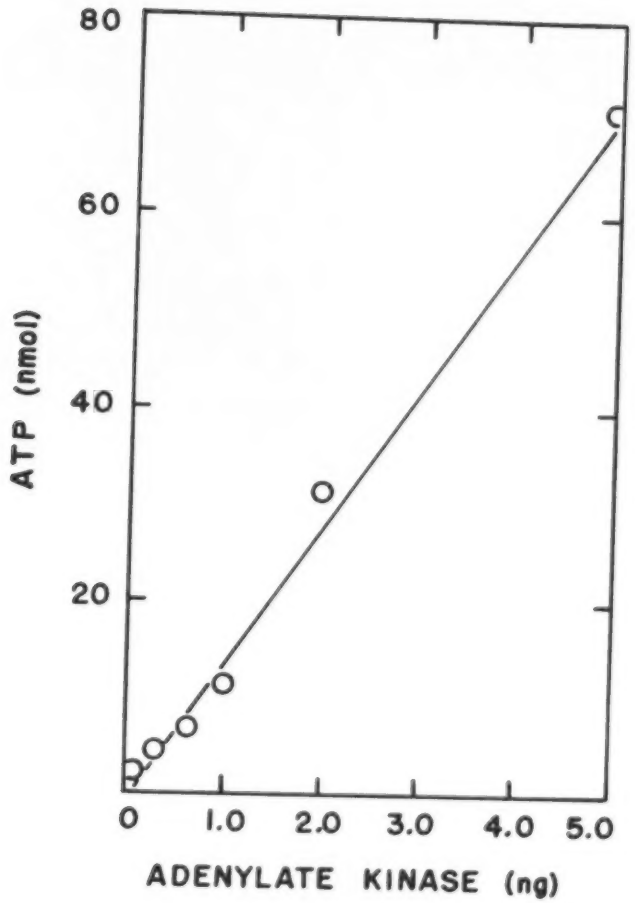


Figure 25. Incubation assay of adenylate kinase. Adenylate kinase samples were treated as in the continuous assay except they were mixed in plastic vials and incubated 1 hr at 37° C. Activity was measured spectrophotometrically at 340 nm and values were reported as ATP production. Reagent blanks were carried through the procedure and subtracted from adenylate kinase samples before being plotted.

c. Measurement of Adenylate Kinase in E. coli

Adenylate kinase was measured in E. coli cells (10^5-10^8/ml) treated with 0.1% toluene using the 1-hr incubation assay. Cell blanks were used to correct for absorbance by cell particles. The limit of detection was 10^6 cells/ml, which corresponds to 0.3 ng adenylate kinase/10^5 cells.

2. Alkaline Phosphatase

Alkaline phosphatase (E.C. No. 3.1.3.1) catalyzes the hydrolysis of a number of natural and synthetic monophosphate esters. Enzymatic activity may be determined by measurement of either phosphate or alcohol (or phenol) production. The sensitivity of the assay can be increased by increasing the incubation time before product measurement.

a. Kinetic Assay.

Alkaline phosphatase activity was determined by measurement of the rate of p-nitrophenol formation spectrophotometrically (Figure 26). The limit of detection was 20 ng of alkaline phosphatase and the range of the assay 0.02-2.0 ug of alkaline phosphatase.

b. Incubation Assays.

i. One-Hour Incubation Assay--Alkaline phosphatase samples (0.1 ml) were mixed with 0.9 ml of the disodium p-nitrophenyl phosphate and incubated at 37° C for 1 hr. p-Nitrophenol was measured spectrophotometrically (Figure 27). The limit of detection was 1 ng and the range of the assay was 1-50 ng alkaline phosphatase. A 1-hr incubation increased the sensitivity of the assay 20-fold over the continuous assay.

ii. Long-Term Incubation Assay -- Incubations of up to 4 d were performed to determine whether the sensitivity of this method for alkaline phosphatase determination could be increased by longer incubations. The disodium p-nitrophenyl phosphate reagent specified for long incubations (Section 5) was used. The time course of p-nitrophenol formation was measured for three different enzyme samples during a 96-hr incubation (Figure 28 top); the proportionality of phenol formation to alkaline phosphatase concentrations after 24-hr and 72-hr incubations is seen in Figure 28 bottom.

The two larger enzyme amounts (8.8 and 0.88 ng) showed a linear response for 3 d but a decreased activity between 72 hr and 96 hr. The smallest enzyme amount tested, 0.09 ng, had a linear response for the 96-hr incubation. Figure 28 bottom shows that a 24-hr incubation gave a linear response over the 100-fold

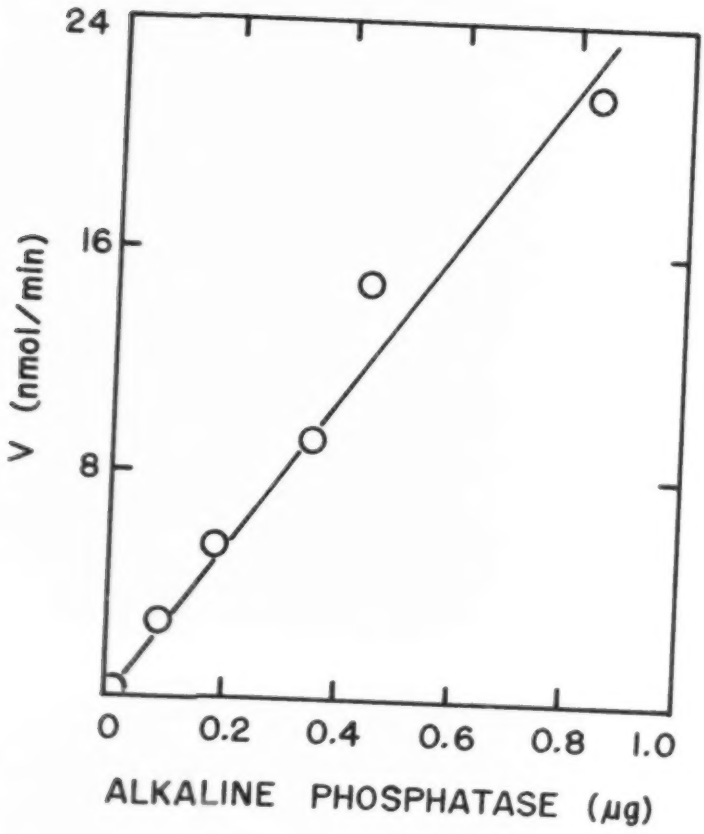


Figure 26. Kinetic assay of alkaline phosphatase. _p-Nitrophenol production was measured spectrophotometrically at 410 nm using a chart recorder calibrated for a full-scale deflection for one absorbance unit. The recorder was operated at 5 cm/min and the minimum change detected was a ΔA of 0.005/min. The sample holder was kept at 30° C for the assays. The molar absorptivity coefficient of _p-nitrophenol is 1.62×10^4 M^{-1} cm^{-1}.

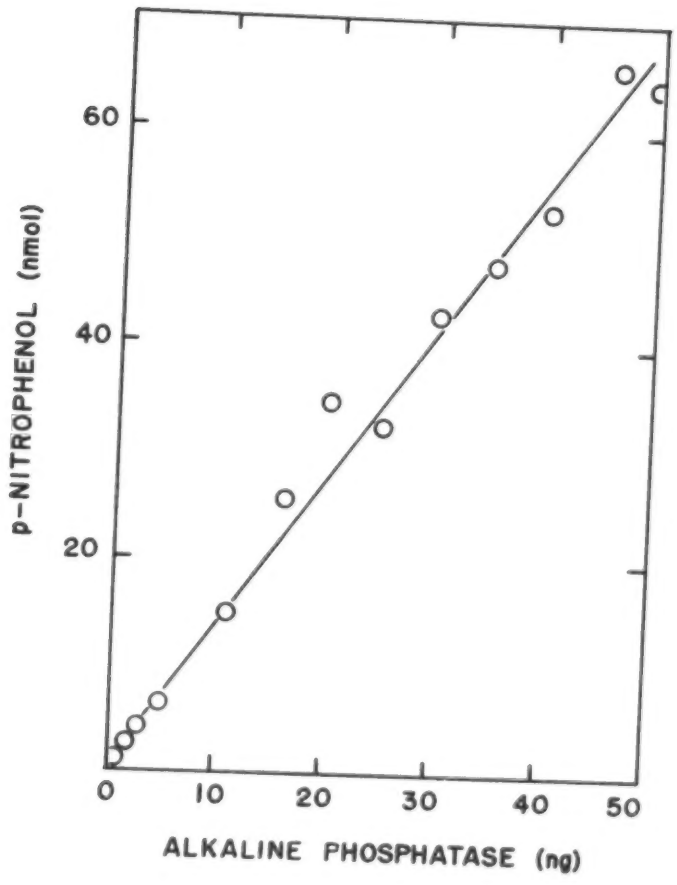


Figure 27. One-hour incubation assay of alkaline phosphatase. p-Nitrophenol production was measured spectrophotometrically at 410 nm after a 1-hr incubation of 0.1 ml samples with 0.9 ml of reagent (0.0066 M disodium p-nitrophenyl phosphate in 0.6 M Tris-HCl, pH 8.2) at 37°C. The values have been corrected for blank hydrolysis of substrate.

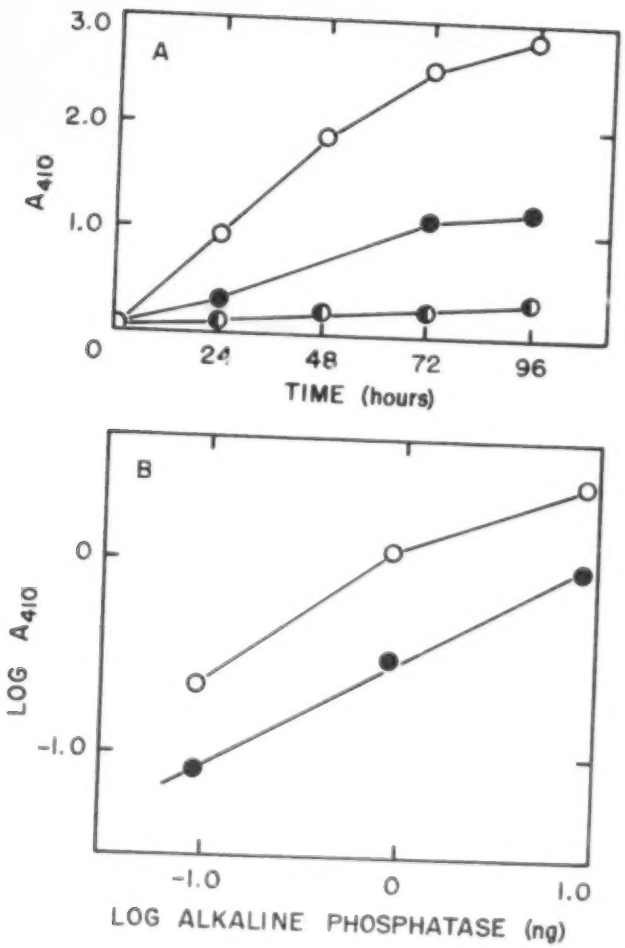


Figure 28. Long-term incubation assay of alkaline phosphatase. Samples (Ø.5 ml) were mixed with Ø.5 ml reagent (Ø.Ø135 M disodium p-nitrophenyl phosphate in 1.Ø M Tris-HCl, pH 8.Ø) in capped plastic vials and incubated at 37°C. Duplicate samples were removed and p-nitrophenol production measured spectrophotometrically at 41Ø nm at 24-hr intervals. Reagent blanks were also read each d so that nonenzymatic p-nitrophenol formation could be determined and used to correct the results of the enzyme samples.
A. Time course of product formation by alkaline phosphatase - 8.8 ng (O), Ø.88 ng (●), and Ø.Ø9 ng (◐), measured at 24-hr intervals for 4 d.
B. Plot of enzyme amounts after a 24- hr incubation (●) and 72-hr incubation (O).

range of alkaline phosphatase tested. Therefore, a long incubation of one or more days can be used to increase the sensitivity of the alkaline phosphatase determination at least 10-fold over a 1-hr incubation assay.

c. Measurement of Alkaline Phosphatase in *E. coli* Cells.

Alkaline phosphatase was measured in *E. coli* cells (8×10^6 - 8×10^9/ml) treated with 0.1% toluene and assayed in an 1-hr incubation assay. Cell samples (0.1 ml) were mixed with 0.9 ml reagent and incubated 1-hr at 37° C. Cell blanks (cell + buffer) were used so that absorbance caused by the cells could be subtracted from the final absorbance readings of the samples. The limit of detection was 8.5×10^6 cells/ml, containing one ng of alkaline phosphatase (from Figure 27).

3. Catalase

Hydrogen peroxide produced by cellular oxidases is scavenged by catalase (E. C. No. 1.1.11.6). Catalase activity is measured by quantitation of residual hydrogen peroxide, either kinetically in a spectrophotometer or after an incubation period.

a. Kinetic Assay

Catalase was measured by spectrophotometric determination (240 nm) of the rate of hydrogen peroxide destruction (Figure 29). A 3-ml assay volume was used because oxygen bubbles produced by the catalase caused fewer problems in 3-ml reaction volumes than in 1 ml. The limit of detection was 0.2 μg and the range tested was 0.2 - 10 μg catalase.

b. Incubation Assay

An 1-hr incubation assay for catalase was used to increase the sensitivity of catalase determination (Figure 30). The amount of hydrogen peroxide utilized was determined by the difference in absorbance between hydrogen peroxide-containing blanks and samples containing catalase. The limit of detection was 1 ng and the range 1-100 ng catalase.

c. Measurement of Catalase in *E. coli*.

Catalase was measured in *E. coli* cells (10^4-10^9 /ml) suspended in distilled water. Toluene treatment was not used since toluene absorbs strongly at 240 nm. An assay was used in which 0.1 ml sample was added to 0.9 ml reagent in capped vials and incubated 1 hr. The limit of detection was 10^7 cells/ml which had the equivalent of 1 ng catalase/10^6 cells.

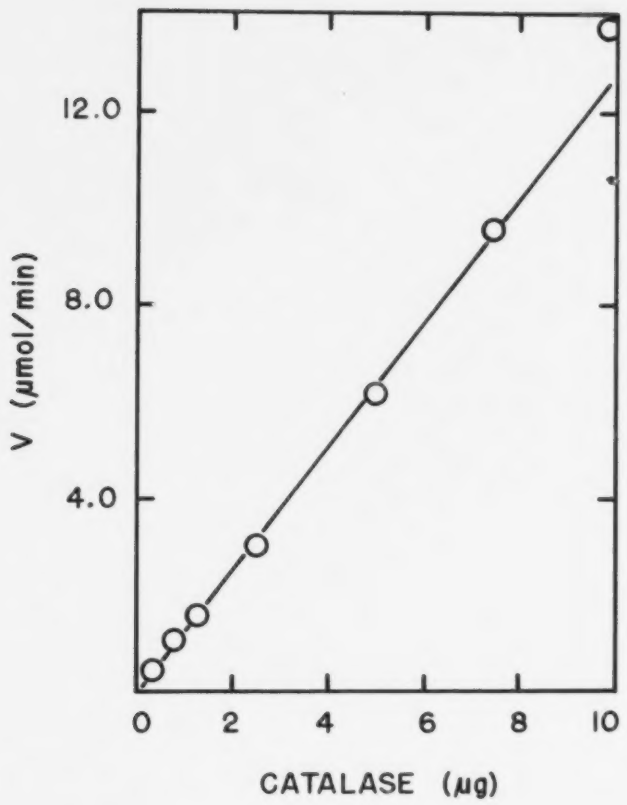


Figure 29. Kinetic assay of catalase. Catalase samples (2 ml) were mixed with 1.0 ml buffered hydrogen peroxide in a 3-ml cuvette and the decrease in absorbance was measured spectrophotometrically at 240 nm using a chart recorder calibrated at 1 absorbance unit/full scale deflection and running at 5 cm/min. The limit of detection was a Δ_oA of 0.005/min. Sample temperature was held at 30° C for the assays.

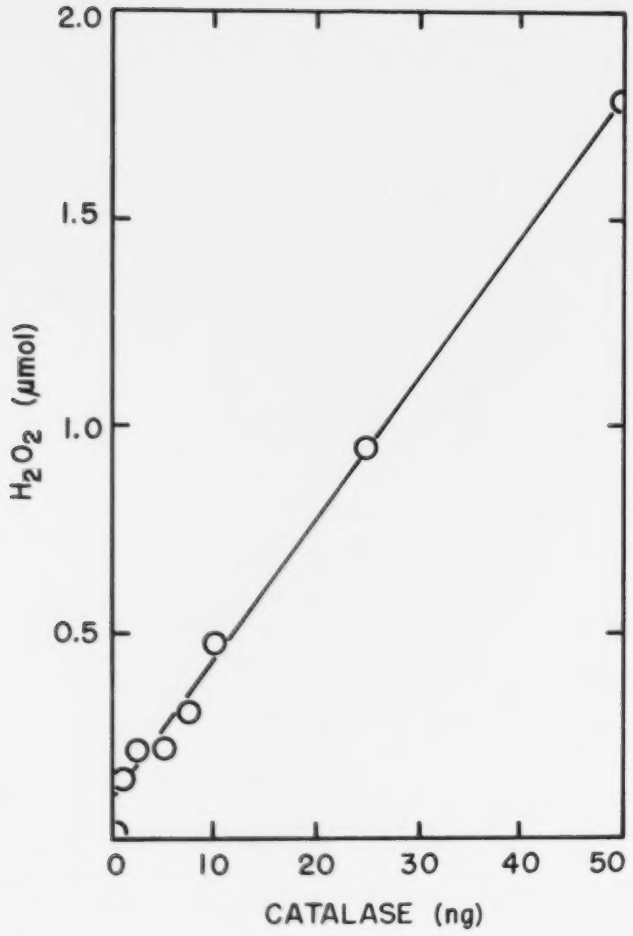


Figure 30. Incubation assay of catalase. Catalase samples (0.1 ml) were mixed with 0.9 ml buffered hydrogen peroxide and incubated 1 hr at 37° C. The hydrogen peroxide used was measured as the difference in absorbance between samples and blanks.

4. Diaphorase

Figure 31 shows the standard curve obtained with diaphorase (E.C. No. 1.6.4.3) assayed as described in the Experimental Procedure section (Section 5).

5. Lactate Dehydrogenase

The glycolytic enzyme lactate dehydrogenase (E. C. No. 1.1.1.27) catalyzes the reduction of pyruvate to lactate and oxidation of NADH to NAD^+. Lactate dehydrogenase from some microbes can use other electron donors such as cytochrome c, ferricyanide, and quinone in addition to NADH, but only the most common substrates, NAD^+ and NADH, were used in this study. In incubation assays, lactate dehydrogenase was coupled to dye reduction by diaphorase, a non-specific enzyme that uses NADH as substrate.

a. Kinetic Assay

Initial velocity measurements of lactate dehydrogenase were made spectrophotometrically by using a chart recorder to monitor changes in absorbance (Figure 32). The limit of detection was 0.1 μg of lactate dehydrogenase or 1.5 nmol NADH oxidized/ min and the range 0.1-15 μg of lactate dehydrogenase.

b. Incubation Assay

A 1-hr incubation assay based on lactate and NAD^+ as substrates was used to measure lactate dehydrogenase. Activity was determined by a coupled enzyme reaction in which NADH was oxidized with concomitant reduction of the dye INT by diaphorase. The use of a dye such as INT or TTC is preferred for many determinations because the product to be measured can be extracted from turbid or soil samples with organic solvents, thus improving the sensitivity of the measurement. INTF formation was measured, both before and after extraction with tetrachloroethylene:acetone (Figure 33). The limit of detection was 1.5 ng and 1.0 ng of lactate dehydrogenase for the unextracted and extracted samples, respectively. The increased response of the extracts, even though the INTF concentration was one-half that of the unextracted samples, was the result of complete solution of INTF in the extract. Precipitation of the water-insoluble INTF was observed in the samples prior to extraction, and this reduced the response for each sample.

c. Measurement of Lactate Dehydrogenase in *E. coli*.

Lactate dehydrogenase was measured in toluene-treated *E. coli* using the incubation assay. Cells (10^6-10^9/ml or 10^5-10^8/assay) were treated with 0.1% toluene prior to addition of

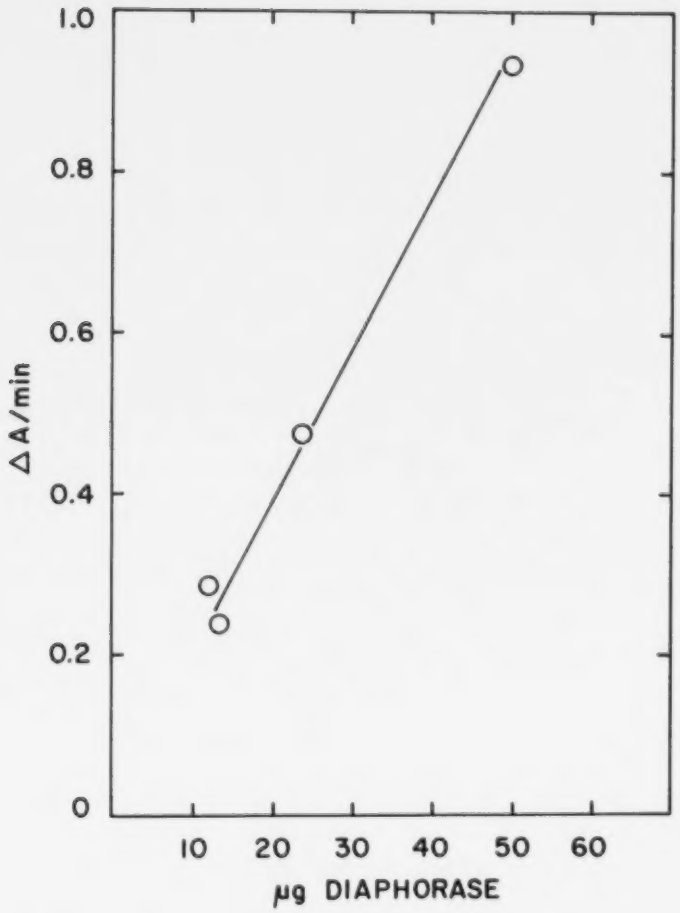


Figure 31. Assay of diaphorase. The reduction INT was measured at 490 nm as described in Section 5.

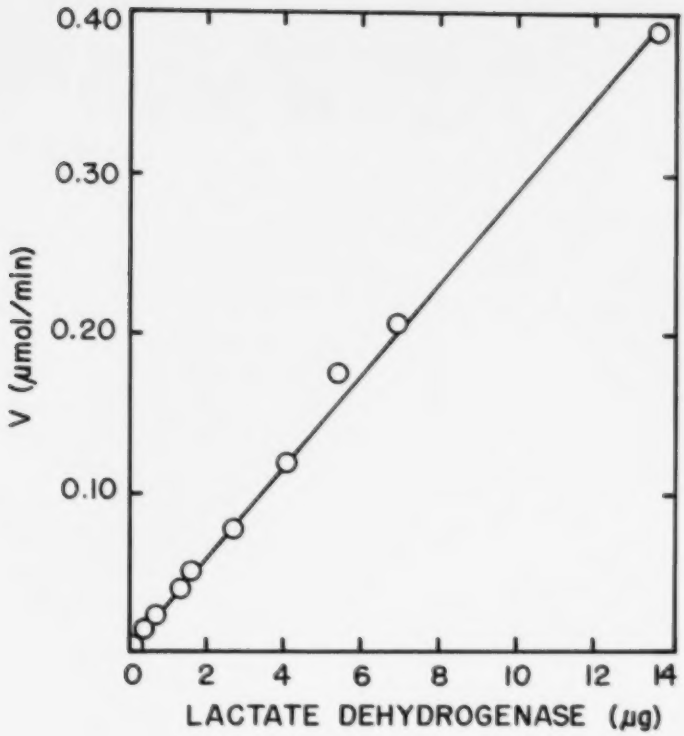


Figure 32. Kinetic assay of lactate dehydrogenase. Absorbance measurements were made at 340 nm. The chart recorder was calibrated so that a full-scale deflection was 1 absorbance unit. The chart speed was 5 cm/min and the minimum change detected was a ΔA of 0.005 A/min. The sample holder on the spectrophotometer was maintained at 30 ^{o}C for the assays.

0.9 ml of the reagent used for the incubation assay. Samples were incubated 1-hr and the INTF produced was extracted with tetrachloroethylene : acetone and read spectrophotometrically at 490 nm. Lactate dehydrogenase activity was observed in all samples, the limit of detection being 10^6 cells/ml. This corresponds to the INTF produced by a 1-hr incubation of 1.0 ng lactate dehydrogenase using the standard curve for extracted samples in Figure 33.

d. Enzymatic Cycling Assay

As stated earlier, the limit of detection for an enzymatic activity is the limit of detection for a change in the amount of substrate or product being measured. An increase in sensitivity of product measurement also increases the sensitivity of enzyme measurement. This concept was tested by using enzymatic cycling to measure NAD^+ produced by lactate dehydrogenase and so improve the sensitivity of detection of the enzyme.

i. Destruction of Excess NADH -- All unreacted NADH must be destroyed prior to enzymatic cyling so that the response will be proportional to the enzymatic activity. This was accomplished by treatment of samples with hydrochloric acid, which destroys NADH without affecting NAD^+, the product of the lactate dehydrogenase reaction. The half-life of NADH at pH 2 at 37° C is 0.07 min so the time required for 99.99% destruction of NADH under assay conditions, using the equation in Table 8, is 0.93 min. The reagent used contained 1 nmol NADH, and a 10-min incubation at pH 2 was used, which was sufficient to decrease the amount of NADH present to 1×10^{-14} mol without changing the amount of NAD^+ produced by the reaction.

ii. Measurement of Lactate Dehydrogenase

Lactate dehydrogenase samples (1pg - 1 ng) were incubated with substrates pyruvate and NADH for 1 hr at 37° C. Unreacted NADH was destroyed by acidification of the samples with hydrochloric acid and by incubation for 15 min. The samples were then neutralized with NaOH and the NAD^+ was amplified by the enzymatic cycling procedure. The results are shown in Figure 34. The limit of detection was 1 pg and the range of the assay was 1 pg-1 ng lactate dehydrogenase. Enzymatic cycling of NAD^+ improved the sensitivity of lactate dehydrogenase detection 1000-fold over the 1-hr incubation assay.

iii. Measurement of Lactate Dehydrogenase in *E. coli* Using Enzymatic Cycling.

Samples containing *E. coli* cells (10^3-10^6/sample) were treated with 0.1% toluene and then lactate dehydrogenase measured by a 1-hr incubation with the pyruvate-NADH reagent followed

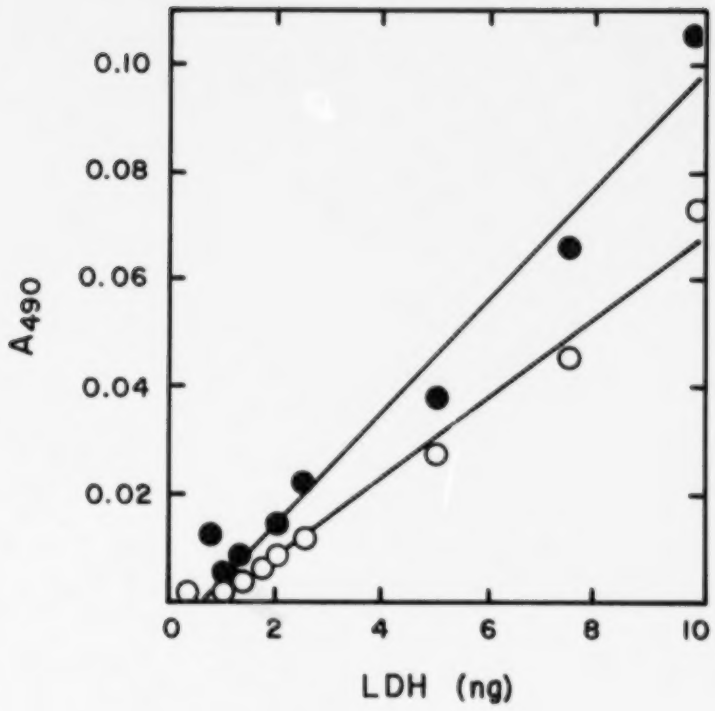


Figure 33. Measurement of lactate dehydrogenase using an incubation assay . INTF formation was measured spectrophotometrically at 490 nm before (O) and after (●) extraction with 2 ml tetrachloroethylene:acetone (1.5:1). Samples (0.1 ml) were mixed with 0.9 ml reagent and incubated 1 hr at 37°C.

TABLE 8. $NADP^+$ DESTRUCTION WITH ALKALI (pH 12.5)

Temperature (°C)	Half-Life (min)	Destruction Time (min) for 99.9%	for 99.99%
38	21.0	209	279.1
65	5.5	54.8	73.0

$$N = N_o \, e^{\frac{-0.693\, t}{t_{1/2}}}$$

where

N = the amount present
N_o = the starting amount
t^o = the incubation time
$t_{1/2}$ = half-life under the incubation conditions

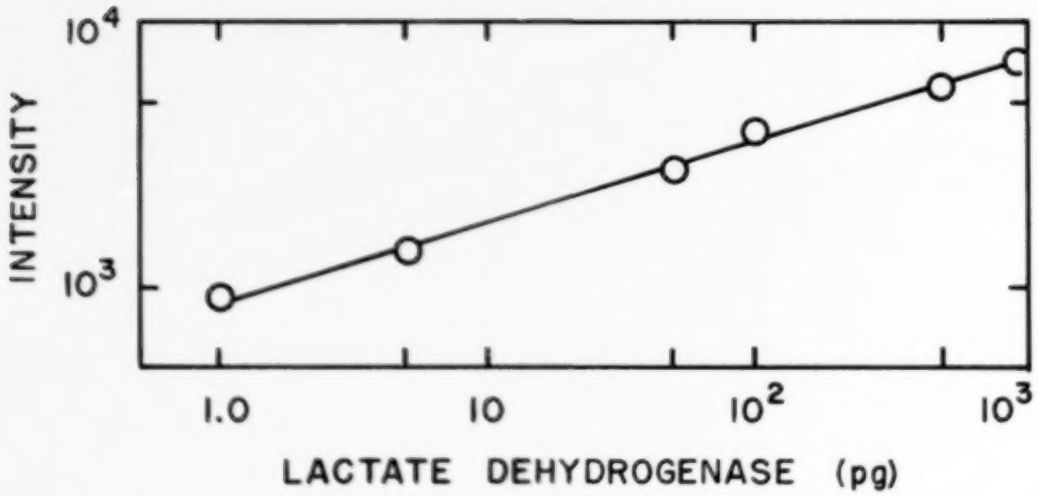


Figure 34. Measurement of lactate dehydrogenase using enzymatic cycling of NAD^+. Picogram amounts of lactate dehydrogenase were measured by enzymatic cycling of NAD^+ produced by a 1-hr incubation of 20 µl samples with 10 µl of LDH reagent (10 nmol NADH and 30 nmol pyruvate in 0.09 M phosphate buffer, pH 7.4) at 37° C. Unreacted NADH was destroyed by treatment with 10 µl 1 N HCl and the NAD^+ was measured by the enzymatic cycling procedure already discussed.

by NADH destruction and enzymatic cycling of NAD^+. Using the lactate dehydrogenase standard curve in Figure 34, the cell samples read as follows:

10^4 cells - 5.4 pg;
10^5 cells - 56 pg;
10^6 cells - 390 pg.

The sample containing 10^3 cells did not have a value above the blanks which were run with the samples. The concentration of the 10^4 cells/assay was 5×10^5 cells/ml. This represented only a two-fold increase in sensitivity of lactate dehydrogenase measurement in E. coli over a 1-hr incubation assay using the dye INT and is not worth the effort required.

6. Microtox

a. Basic Measurements

Figure 35 shows the standard curve for determining the toxicity of toluene with the Microtox assay. The experiment was done as described in Section 5. The EC_{50} is 50 ppm. Table 9 shows the EC_{50} values for a number of compounds and the broad range of concentration of inhibitors that can be measured in the system--ranging from 47×10^3 ppm for ethanol to 2.5 ppm for cyanide. Although the light output from a sample of bacteria was reduced to 1/3 during 5 hr of storage after hydration, the EC_{50} values determined for m-cresol with fresh and 5-hr-old samples were 11 and 12 ppm, respectively. An example of the reproducibility of experiments is that with a concentration of 9 mg/L of m-cresol the normalized light loss was 44.0% for the first experiment and 44.9% for the second.

b. Environmental Samples

The results of typical application of the Microtox method to environmental samples are shown in Table 10. In Part A various local water sources were tested, and in Part B various spring water samples from eastern Oklahoma were tested. The amounts of lipopolysaccharides present in these samples as determined by the Limulus amebocyte lysate assay are also shown. Part C shows the results of testing various oil refinery effluents. The LC_{50} for the fathead minnow 96-hr bioassay is also shown for some.

c. Toxicity of Pesticides

A number of commonly used pesticides were tested in the Microtox bioassay (Table 11). The luminescent bacteria were fairly sensitive to most of the compounds tested. Thus, the Microtox assay is an effective method for their determination.

The sensitivity with which light production can be measured with commercially available instrumentation and the availability

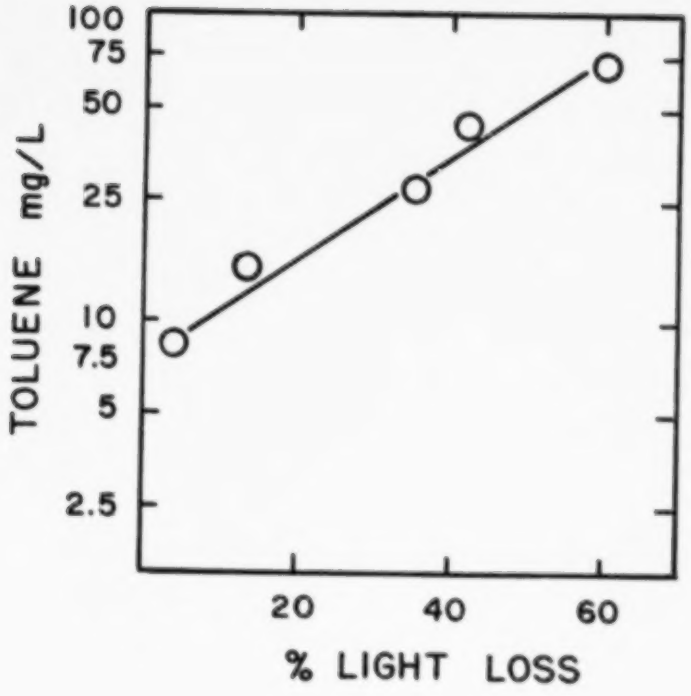


Figure 35. Standard curve for toxicity of toluene with the Microtox assay. The inhibition of light production by various concentrations of toluene was determined as described in Section 5. The logarithm of toluene concentration is plotted against the per cent of light production loss by the luminescent bacteria.

TABLE 9. TYPICAL EC_{50}, LD_{50} and LC_{50} FOR SELECTED COMPOUNDS

Typical Compounds	Microtox EC_{50} ppm	Rat (oral)[1] LD_{50} g/kg	Fish toxicity LC_{50} mg/L
Ethanol	47,000	14	13,000[2]
1-Butanol	44,000	4.4	1,900[2]
Benzene	200	5.7	50[2]
Toluene	50	5.0	23[2]
Phenol	26	0.53	5.0[2]
m-Cresol	11	2.0	19 (p cresol)[2]
Formaldehyde	8.7	0.80	250[3]
Respiratory Inhibitors			
Amytal	1,000		
Thenoyltrifluoroacetone	3.5		
Cyanide	2.5		
Azide	400		
Arsenate	94		

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[1] Lethal dose $_{50}$. From the Merck Index, Merck & Co., Rahway, N.J.

[2] Lethal concentration $_{50}$ BRUNGS, W. A., J. H. McCORMICK, T. W. NEIHEISEL, R. L. SPEHAR, C. E. STEPHAN, and G. N. STOKES: J. Water Poll. Control 49, 1425 (1977).

[3] McKIM, J. M., R. L. ANDERSON, D. A. BENOIT, R. L. SPEHAR, and G. N. STOKES: J. Water Poll. Control 48, 1544 (1976).

TABLE 10. APPLICATION OF MICROTOX TO ENVIRONMENTAL SAMPLES

Water Sample	Microtox Assay LL_2	LL_4	Lipopolysaccharide pg/mL
A. Local Water			
Barstead still system	15	27	$5.0 \times 10^{2}_{5}$
Burned field water	100	100	1.0×10^{5}
Essenberg well	0	2	75
Glass-distilled water	0	0	0
Spring(obtained locally)	93	96	$1.0 \times 10^{4}_{2}$
Tap water	35	35	$4.4 \times 10^{2}_{2}$
Water fountain	2	7	5.0×10^{2}
B. Eastern Oklahoma			
Park Spring	0	0	$5.0 \times 10^{4}_{7}$
Sparrow Hawk Spring	-15	-15	2.5×10^{7}
Speed's Spring House	-24	-20	-
Stream	0	-1	2.5×10^{4}
Wilson Ranch Spring	-14	-14	-

	Percent required for Microtox LL_4	EC_{50}	Fathead Minnow LC_{50}
C. Oil Refinery Effluents			
ETE - 55	46	58	65
17 - 51 - 80	34	74	65
LNX	0	100	65
UQB - 3	90	1.8	42
UQB - 4	0	100	75

The sample to be tested constituted 45% of the Microtox assay volume. For the EC_{50} determination in part C the concentration of the effluent was varied.

TABLE 11. MICROTOX TOXICITY OF SELECTED PESTICIDES

Common Name of Compound	EC_{50} ppm Microtox	LD_{50} mg/kg[1] Rat (oral)
Captafol	7	6200
Carbaryl	2	500
Cyhexatin	10	540
Diazinon	1.7	300
Dichloran	3	5000
DDT	7	110
Glyphosate	7.7	4300
Malathion	10	1400
Paraquat	780	150
Ridomil	120	670
Thiabendazole	3400	3100

[1] From Farm Chemical Handbook, 1980, Meister Publishing Co., Willoughby, OH.

of suitable freeze-dried preparations of luminescent bacteria enhance the usefulness of the Microtox bioassay. The ease of performance of the basic measurement and the range of concentrations of toxic and other compounds that can be determined are also attractive features of the Microtox bioassay.

Table 9, Part A shows a variety of compounds with differing toxicities assayed in three systems. These three measures of toxicity have good correlations. The Microtox has a correlation coefficient of 0.90 with the rat toxicity and 1.0 with the fish toxicity. Advantages of the Microtox test are the short time (30 min) required for an assay and the statistical advantage in using over 10^9 bacteria instead of a small number of rats or fish in the other bioassays. Because the luminescent pathway is a branch of the electron transport chain, the effects of various respiratory inhibitors were determined. The relationship between the electron transport chain and the luminescent pathway would suggest that inhibitors acting after the divergence of the two pathways would be less effective in blocking light production and even increasing it. This is not observed; therefore, the metabolic interrelationships must be more complicated.

The local water samples varied greatly in both their inhibition of light production and in their lipopolysaccharide content. There is no correlation between these two parameters, but there is no reason to expect that there should be. Some of the eastern Oklahoma spring waters stimulate light production (shown by the minus sign). Certain of the compounds (at low concentrations) such as ethanol also stimulate light production. These compounds could either be substrates or change the availability of components.

Table 10 shows little relation between light inhibition by selected pesticides and their LD_{50} orally for the rat. The correlation coefficient between these variables is 0.09.

G. Lipopolysaccharide Determination

Limulus Amebocyte Lysate Assay

The unusual reaction of the hemocyte lysate from the horseshoe crab in which a proclotting enzyme is activated by lipopolysaccharides (LPS) producing a clot has been exploited in several biomedical applications (39). Since the lipopolysaccharides of gram-negative bacteria are pyrogenic, and are endotoxins, their detection has great clinical significance. Sullivan, Valois, and Watson (94) have reviewed the development of the Limulus amebocyte lysate system (LAL) as a test for endotoxins.

A method for the detection of as little as 100 pg of endotoxin has been described by Jorgensen and Smith (95). A slide test has been developed which reduces the volume required (Frauch, (96) and Flowers (97)).

Watson, Novitsky, Quinby and Valois (98) have used the LAL

determination of LPS as an indirect assay of the number of bacteria in the marine environment. The biochemical basis of the clotting has been established through the studies of Liu and coworkers (99-101). A MW of 150 K was found for the proclotting enzyme which, when activated by Ca^{2+} and endotoxins, is a serine protease (possible MW 79-84 K). The purified coagulogen has a MW of 24.5 K; when acted upon by the clotting enzyme, it is converted to a 17.5-K protein and a 5.5-K peptide by hydrolysis at a single arginine-lysine linkage. There is a LPS binding protein of MW 80 K which is found on the amebocyte membranes.

Correlation ofthe LAL test with numbers of coliform, enteric, gram-negative, and heterotrophic bacteria was found by Evans, Schillinger, and Stuart (102) for stream water. Goto and Nakamura (103) used a dry-up method on a glass slide to reduce the volume of LAL needed. Picogram quantities of LPS were measured with (^{125}I)-labeled coagulogen by Munford (104); this procedure also required less LAL.

An alternative assay using a chromogenic peptide substrate for the active clotting enzyme was useful over the LPS range of 0.2 to 10 ng/ml (105).

Figure 36 shows the standard curve that we obtained when LPS-induced coagulation was measured photometrically. Using E. coli cells as the standard with their number being determined by plate counts and turbidity measurements, we obtained the results shown in Figure 37.

H. ATP Determinations

Adenosine triphosphate may be measured directly with firefly luciferase or indirectly with a coupled enzyme system using NADPH for spectrophotometric or fluorometric determination. Enzymatic cycling can be used to amplify the response and increase the sensitivity of measurement of ATP.

1. Coupled Enzyme Assay

A coupled enzyme reaction of hexokinase-glucose 6- phosphate dehydrogenase was used to measure ATP by the fluorescence of NADPH. Tests of the assay using both ATP and glucose 6-phosphate standards showed that the reaction was complete and that all ATP was used up in less than 5 min at 37°C. A typical standard curve is shown in Figure 38. The limit of detection was 2×10^{-10} mol and the range 2×10^{-10}-2×10^{-8} mol of ATP.

2. Enzymatic Cycling

The sensitivity of detection of ATP was improved by enzymatic cycling of the NADPH produced by the coupled enzyme assay. Excess $NADP^+$ in the coupled enzyme reagent was destroyed by alkali treatment prior to cycling. This approach was selected instead of an ADP/ATP cycle (Figure 39) because the $NADP^+$

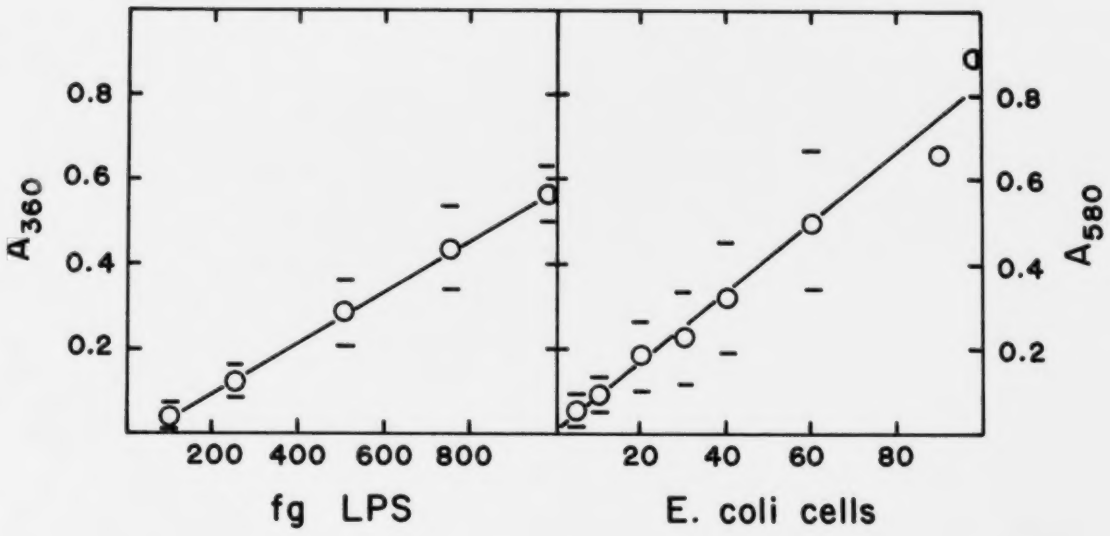

Figure 36. <Left> LPS standard curve. The Limulus amebocyte lysate assay for lipopolysaccharide was performed as described in Section 5. The reaction was followed by the spectrophotometric assay.

Figure 37. <Right> E. coli determination using LAL. The results of the more sensitive LPS procedure with a bromosulfalein determination of the gelled protein are shown. The E. coli cells were diluted by another worker so that the determinations were not prejudiced. The procedure is described in Section 5.

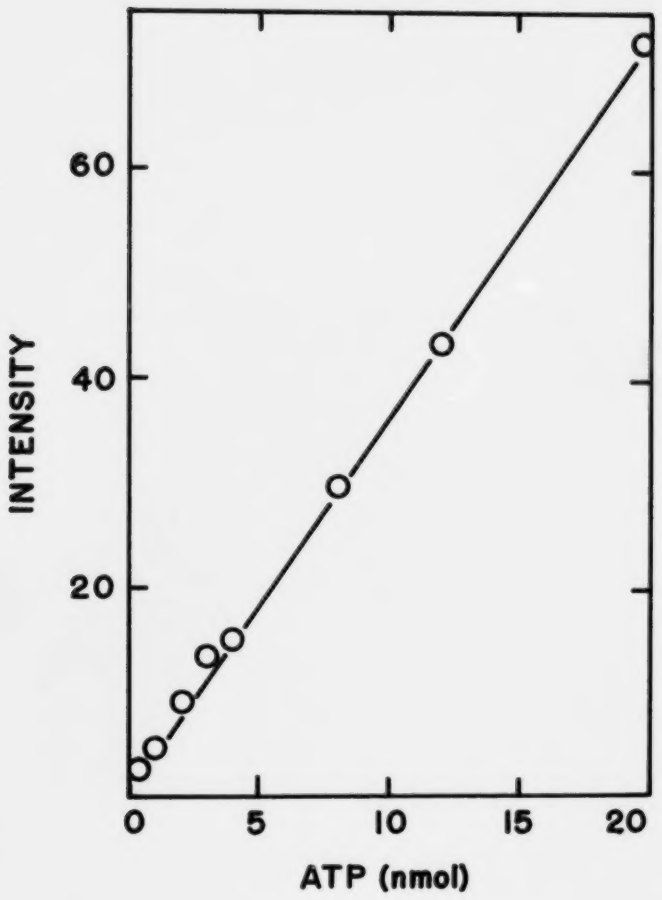

Figure 38. Coupled enzyme assay of ATP. ATP was measured by reaction with hexokinase and glucose-6-phosphate to produce NADPH, which was measured fluorimetrically (excitation 340 nm, 1-mm slit; emission 455 nm, 2-mm slit). ATP samples (0.1 ml) were mixed with the coupled enzymes reagent (0.9 ml) and incubated 15 min at 37°C.

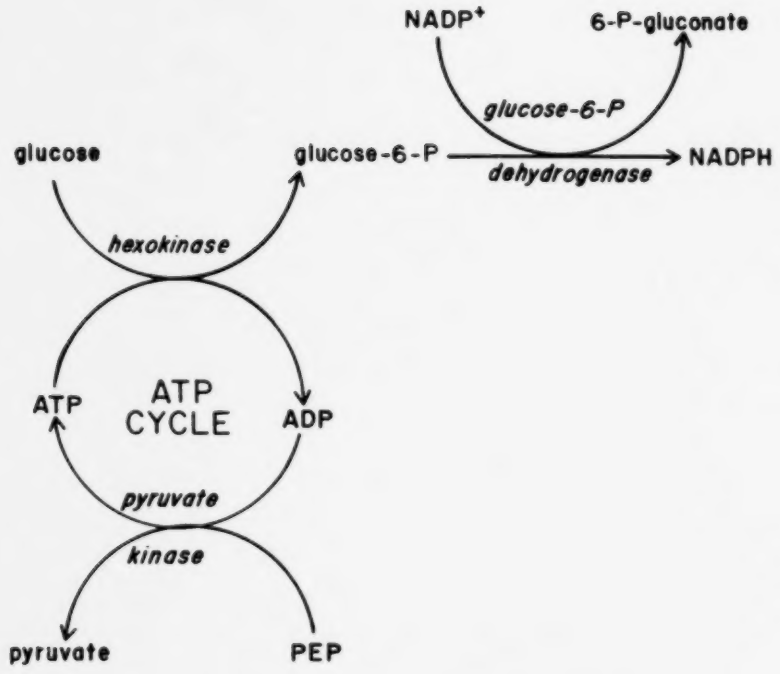

Figure 39. Reaction scheme for the enzymatic cycling of ATP.

cycling procedure had been developed and could be applied to the measurement of another metabolite, ATP.

a. Destruction of Excess $NADP^+$

For enzymatic cycling to be useful, all unreacted $NADP^+$ must be destroyed so that only the NADPH formed by the coupled enzyme assay of ATP participates in the cycling reactions (Figure 40). This was accomplished by alkali treatment prior to addition of the cycling reagent, since $NADP^+$ is labile at high pH (Table (8)). A 99.99% destruction of $NADP^+$ was accomplished by a 1-hr incubation of samples at pH 12.5 (65°C) which means that 5×10^{-13} mol of $NADP^+$ remained of the 1×10^{-9} mol in the reagent after treatment. The residual $NADP^+$ was a major contributor to the blank fluorescence and thus largely determined the sensitivity of ATP detection.

b. ATP Measurement by Enzymatic Cycling

ATP was measured using enzymatic cycling of NADPH after alkali treatment (Figure 41). The limit of detection was 2×10^{-13} mol and the range 2×10^{-13} to 2×10^{-11} mol of ATP. $NADP^+$ standards produced a parallel curve but with a lower blank and so a greater sensitivity. The limit of detection of ATP by enzymatic cycling was improved 1000-fold over the coupled enzyme assay method.

3. Measurement of ATP in *E. coli*

ATP was extracted from *E. coli* using boiling Tricine buffer and measured by the enzymatic cycling procedure discussed earlier. The extraction procedure yielded 90% of the ATP in standard samples so no correction was made for recovery. Since extracts contained ATP and pyridine nucleotides, the response was the sum of ATP and reduced pyridine nucleotides.

When 5×10^7 cells were extracted with 5 ml boiling Tricine buffer and 0.02-ml portions of the extract were used in the cycling procedure, there was 5.4×10^{-12} mol of ATP equivalent/20 µl. This may be expressed as 1.35×10^{-9} mol ATP and reduced pyridine nucleotides / 5×10^7 cells. When the amount of the pyridine nucleotides were subtracted, the ATP content was 1.23×10^{-9} mol/5×10^7 cells.

4. Bioluminescent ATP Assay

The bioluminescence produced when firefly luciferase (EC 1.13.12.7) reacts with ATP, luciferin, and oxygen is the basis of an extremely sensitive method for ATP determination. A whole technology has developed (Leach, F.R., Review in preparation). We examined a number of procedures for ATP determination using firefly luciferase (1). Because of the diversity of conditions

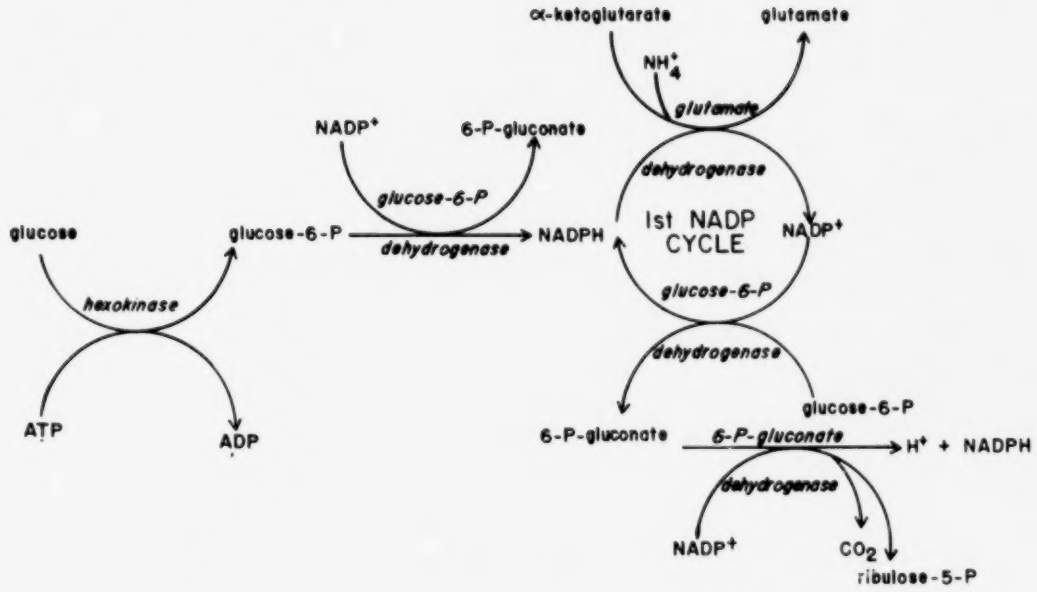

Figure 40. Reaction scheme for the measurement of ATP using enzymatic cycling of $NADP^+$.

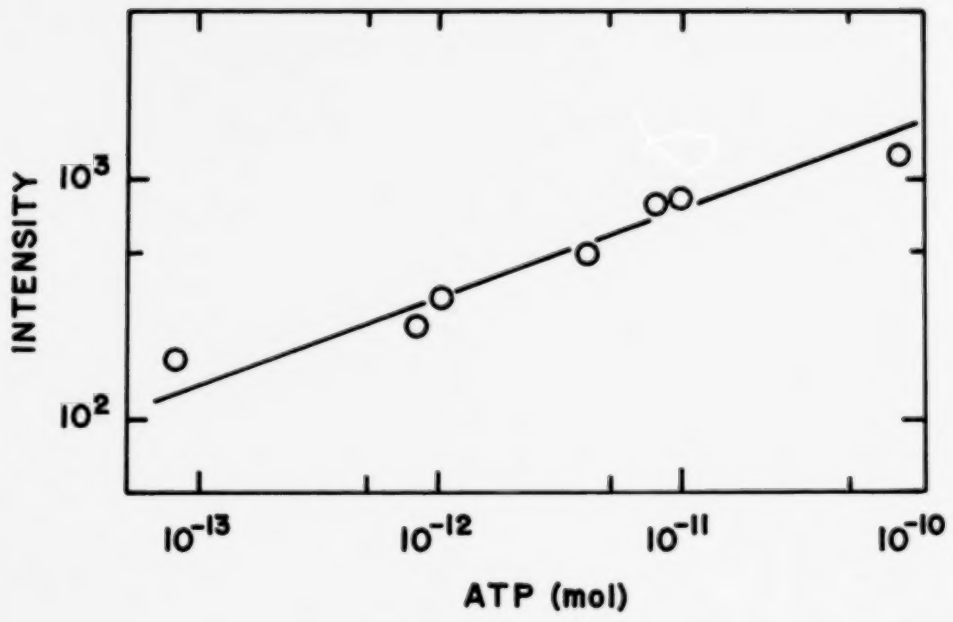

Figure 41. Enzymatic cycling assay of ATP. ATP was measured by enzymatic cycling of NADPH produced by the coupled enzyme reactions discussed earlier (Figure 17). Samples (20 µl) were incubated with 10 µl of coupled enzyme reagent 15 min, treated with NaOH and incubated 1 hr at 65° C, neutralized, and carried through the standard $NADP^+$ cycling procedure.

used and the current availability of a number of commercial luciferase preparations, we optimized the assay (44) and compared the properties of the various commercial preparations (41). Conditions were established for the determination of as little as 50 fg of ATP (43). Tricine was found a superior buffer (42). Since these publications contain the details of much of our work on ATP analysis, we will present only minimal information here.

Figure 42 shows a typical standard curve obtained by the procedure described in Section 5. The position of the curve can be varied depending upon the amounts of luciferase and luciferin used (42). The details of optimizing the assay are in the paper by Webster and Leach (44).

5. Comparison of Enzymatic Cycling and Bioluminescent Assays

Since this laboratory has studied ATP determination both with firefly luciferase and with enzymatic cycling, a comparison of the two methods for the determination of ATP was made (Table 12). The parameters compared were range, sensitivity, cost, productivity (number of samples measured, not counting standards), inhibitors, equipment, turnaround time (time required to get a result and repeat an assay), and specificity. The firefly luciferase was clearly as good or better in every category except inhibitors, which is of vital importance in the measurement of environmental samples. Enzymatic cycling could be used for those samples that contain inhibitors preventing determination by firefly luciferase; as such it is a useful procedure.

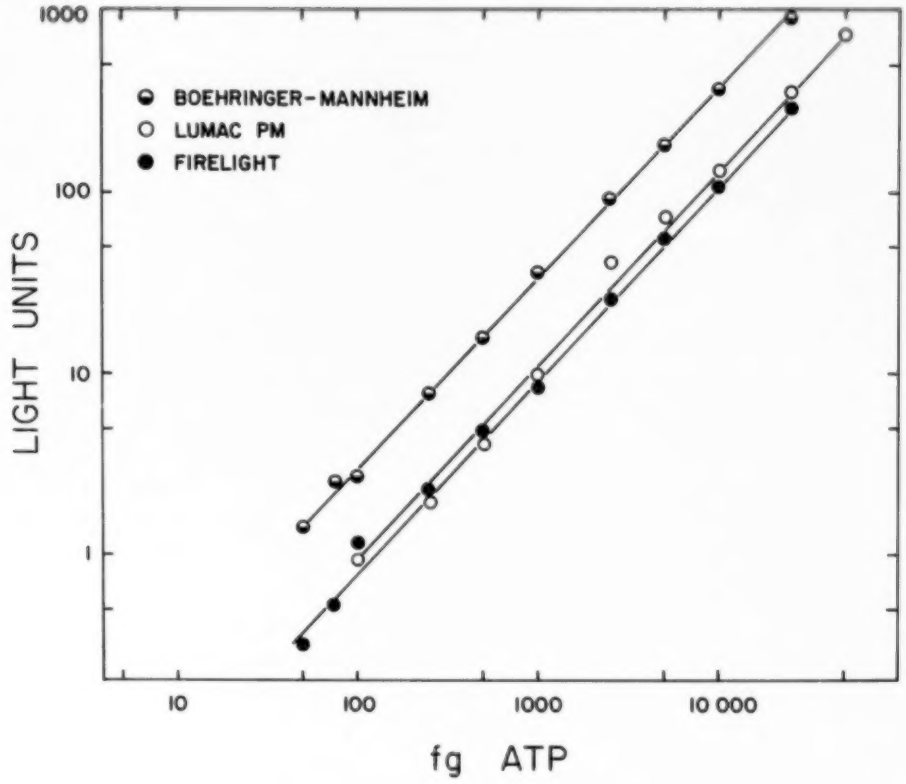

Figure 42. Standard curve for ATP determination by firefly luciferase. Light production is determined using a Packard Model 6100 photometer maintained at 25 ± 0.1° C. The light production without added ATP is subtracted. The reaction system (0.2 ml) contained 0.025 M Tricine buffer, pH 7.8, 5 mM $MgSO_4$, 0.5 mM EDTA, and 0.5 mM DTT. With Boehringer-Mannheim luciferase, 100 µg of bovine serum albumin and 50 µg of luciferin were added. Various amounts of ATP contained in 20 µl were injected into the cuvette and the light emission was counted for 30 sec after a 1-sec delay. The following luciferases were used: Lumac's Lumit PM Lot Nos. 5041 and 5045 (130 units), Analytical Luminescence Laboratory's Firelight Lot No. 6621 (102 units), and Boehringer-Mannheim luciferase Lot No. 1210301 (361 units).

TABLE 12. COMPARISON OF ATP MEASUREMENT TECHNIQUES

Parameter	Luciferase	Cycling
Range[a]	0.2 pmol - 100 pmol	0.3 pmol - 10 pmol
Sensitivity[b]	0.2 fmol (0.1 pg)	0.1 pmol (50 pg)
Cost[c]	6 cents/assay	9.5 cents/assay
Productivity	25/hour or 200/8-hour day	96/5 hours or 192/8-hour day
Inhibitors	Metal ions, PO_4^{-3}	None encountered to date[d]
Equipment	Photometer	Fluorimeter
Turnaround Time	<30 minutes	5 hours
Specificity	ATP only	ATP, NADH, NADPH

[a] Useful range of ATP amounts which can be routinely measured.
[b] Smallest amount of ATP detected by the assay.
[c] Based on 1979 prices.
[d] An optimistic statement.

SECTION 7

ENVIRONMENTAL SAMPLES

Since the goal of the research reported herein is applicability to environmental samples, the described procedures were tested with such samples. As described previously in Section 5, several of the procedures were eliminated because they were 1) not sensitive enough, 2) involved complicated procedures, or 3) required equipment not commonly available. For the remaining procedures, techniques were developed for their application to environmental samples. These procedures and their application are now described.

A. ATP Extractions

1. Comparison of Procedures

Many methods are available for the extraction of ATP from various types of environmental samples. The media for the initial extraction can be divided into four categories: 1) aqueous buffers, 2) inorganic acids, 3) organic acids and other solvents and 4) commercially available preparations (Table 13). We have applied many of these extractants to produce ATP solutions and utilized those that seemed the most promising to liberate ATP from environmental samples.

In our hands boiling Tricine containing Mg-EDTA seems to be the simplest and one of the more effective methods for microbial cells. Sulfuric acid and dimethyl sulfoxide are effective for soil samples and cells. Several commercial releasing agents, NRB Reagent from Lumac and Releasing agent N (cat # 04-40-40) from SAI, were not satisfactory when the manufacturers' recommended procedure was followed. Some preliminary experiments indicate that centrifugation to remove the soil and analysis in the Pico-Lite luminometer might make them applicable. We have not used the commercial releasing agents for microbial cell ATP extraction.

The detailed procedures for boiling Tricine, DMSO and sulfuric acid extractions of ATP are found in Section 5.

TABLE 13. METHODS AVAILABLE FOR EXTRACTION OF ATP

Media	Condition for Extraction Concentration M	Temp C	Time Min	Recovery[1] %	Reference
A. Aqueous Buffers					
Tris	0.02 or 0.05	100	2	59-65	(106)
Tricine	0.05	100	2	59-63	(42)
Tricine-EDTA	0.05-0.002	100	1.5	93	
Tricine-Mg	0.05-0.01	100	1.5	82	
Tris-EDTA-Mg	0.05-0.002-0.01	100	1.5	89	
Tris-EDTA	0.05-0.002	100	1.5	100	(107)
Glycylglycine-EDTA	0.05-0.002	100	1.5	100	(107)
Phosphate-EDTA	0.05-0.002	100	1.5	100	(108)
Water	Tap-distilled	100	2	87-100	(109,110)
B. Organic Acids and Solvents					
Ammonium Formate	1	25		12-21	
n-Bromosuccinimide-EDTA	0.01-0.01	25		62-96	(111)
Chloroform (saturated with aqueous)					
$CHCl_3$-Tricine	100%,0.1	25		78	(112,113)
$CHCl_3$-$NaHCO_3$	100%,0.5			35-46	(114)
$CHCl_3$-PO_4^{-3}	100%,0.01			63-72	(115,116)
Dimethylsulfoxide	90%	25		48-65	
Dimethylformamide	90%	25		17-20	
Methanol	100%	100		59-94	
Ethanol	95%	100		64-86	(117)
1-Butanol	100%	100		64	
2-Butanol	100%	100		68	
Octanol-Butanol	1:8 v/v	25		71-99	(118)
TCA	10%			3-13	
TCA-EDTA	0.51-0.017			9	(107)
C. Inorganic Acids					
Sulfuric Acid	0.6 N	0		80	(112,119)
	1.5 N	0-4		-	(120)
Nitric Acid	0.1 N	0		-	(121)
Perchloric Acid	0.4	0		-	(110)

[1] Based on ATP standard solutions with data obtained in this lab.

2. Optimizing Conditions

Many reagents are capable of extracting ATP from environmental samples. However, we have found that many of these reagents subsequently interfere with the assay and consequently must be removed. Sample size, type, concentration, and volume of extracting reagent, methods of mixing, temperature, etc., need to be considered in developing an optimum extraction method.

The components of the culture medium for cells interfered with the assay; this interference was eliminated by harvesting the cells by centrifugation and washing twice with buffer or minimal medium. If heat is used for cell lysis the temperature must exceed 96° C inside the test tube for complete extraction. Heating the tubes prior to addition of the cells and injecting a small volume of cells (usually 1 part into 10) into the buffer helps to accomplish this. If boiling buffers are used for extraction additional EDTA enhances the yield of ATP from solutions; if cells are extracted, additional sensitivity is achieved by adding both Mg^{2+} and EDTA.

Dimethyl sulfoxide is a very good extractant for cellular and soil ATP. The method is easy and very quick. The major drawback is the inhibition of the luciferase assay by small amounts of DMSO. This inhibition can be seen in the data presented in Table 14. If the samples contain enough ATP to be diluted 10- or 100-fold the DMSO inhibition can be effectively eliminated.

Sulfuric acid is an excellent extractant for ATP from soil and cells. Many investigators use 0.6 N H_2SO_4, but this concentration is too low for some types of alkaline soils, so we use 1.5 N H_2SO_4. For the soils that we have tested 5-10 ml of acid per gram of soil is optimum, and samples of from 1-5 g are adequate. We use 2 g of soil/10 ml acid. The use of a Polytron and wrist-action shaker facilitate the extraction of ATP from larger soil samples (1.3-fold increase in activity for each separately and 2.3-fold increase in measured ATP activity when both are used) by aiding in the breakdown of soil and allowing good mixing.

The greatest difficulty encountered with the use of acids for extracting ATP is the neutralization of these extracts prior to analysis since the luciferase assay is very pH sensitive. Acids also extract unknown compounds from the soil which inhibit the assay. We have used strong bases (NaOH, KOH, ethanolamine), buffers containing strong bases, 0.5 M Alamine in Freon, cation exchange columns, charcoal adsorption, and dilution as a means of neutralizing and/or cleaning up acid extracts. Experiments have shown that compounds of high ionic strength, ethanol, and Alamine/Freon are inhibitory so we have avoided using them for neutralization. The acid extract of soil is diluted 1:10 with buffer, a small aliquot of the diluted extract is taken, and the pH adjusted to 7.5 with 10 N KOH or 5 N ethanolamine using phenol red indicator (0.1%). From this the amount of base required to

TABLE 14. DMSO INHIBITION OF FIREFLY LUCIFERASE

DMSO %	Inhibition %
Ø.23	2
Ø.9	21
4.5	39
9.Ø	59
18.Ø	86

The % of DMSO is the final concentration in a 1-ml reaction volume.

neutralize 1 ml of extract can be calculated.

Inhibitory compounds extracted with the ATP can be removed by cation exchange and charcoal adsorption. These methods will be discussed later.

3. Stability of Samples

Soil samples were stored in sterile test tubes at three temperatures, 25°, 4°, and -15° C, to determine the optimum method of treating them before extraction. Frozen samples (-15° C) retained ATP activity equivalent to samples extracted initially for 16 wk. Samples stored at 4°C showed more variation from week to week than frozen samples but retained 81% of their initial activity. Samples stored at room temperature (25° C) retained only 22% of their activity for 1 wk. These room-temperature samples showed a 122-147% increase in activity during the first 72 hr suggesting that this could perhaps be used as an amplification technique. These experiments all used the sulfuric acid-Tricine dilution method for extraction.

The stability of sulfuric acid extracts of soil, ATP solutions and soil spiked with ATP was monitored for 12 wk. The soil and ATP solutions showed no significant decrease in activity over the 12 wk when extracted, diluted 1:10 with 0.1 M Tricine, pH 9, neutralized with 5 N ethanolamine and stored frozen. The spiked soil samples were more variable, probably due to adsorption of the ATP by the clay type soil used.

Tricine extracts of E. coli were stable for 1-2 wk when stored frozen.

4. Recovery of ATP

Three different sources of ATP were used to determine recovery when the various extraction methods were applied to solutions, cell suspensions, or soil samples. These sources were a known quantity of ATP in solution, (^{3}H) -ATP, and a known quantity of E. coli cells. These results are shown in Table 15. These results suggest that further research is required to optimize extraction and recovery procedures.

5. Purification Using Charcoal and Ion Exchange

One of the major difficulties encountered in measuring ATP extracted from soil is the concurrent extraction of several unknown inhibitors of the firefly luciferase reaction. Dilution techniques, charcoal adsorption of the ATP, and cation exchange have been studied using (^{3}H)-ATP Extract cleanup was tested with polypropylene columns containing charcoal, Amberlite or Dowex. The (^{3}H)-ATP is retained (97-99%) on the Amberlite IR-120, charcoal or Dowex 50 after one pass. The cation exchange columns do not release the ATP readily without the use of eluants of high ionic strength (5 N NaOH for Amberlite and 1 M sodium

TABLE 15. RECOVERY OF ATP USING VARIOUS EXTRACTION METHODS

Method	Sample	Spike	Recovery %
A. Boiling Tricine			
0.05 M, 2 min	Solution	ATP standard solution	63
0.05 M, 90 sec	Solution	ATP standard solution	126
Tricine-EDTA	Solution	ATP standard solution	104
Tricine-Mg-EDTA	Solution	ATP standard solution	99
Tricine	Solution	ATP standard solution	97
0.05 M	E. coli	ATP standard solution	93
Tricine-EDTA	E. coli	ATP standard solution	75
Tricine-Mg	E. coli	ATP standard solution	82
Tricine-Mg-EDTA	E. coli	ATP standard solution	89
Tricine-Mg-EDTA	B. subtilis	ATP standard solution	53
Tricine-Mg-EDTA	Soil	ATP standard solution	0
B. Butanol-octanol	E. coli	ATP standard solution	98
C. Dimethyl sulfoxide	Solution	ATP standard solution	59
	E. coli	ATP standard solution	31
	Soil	ATP standard solution	7
D. TCA-EDTA	E. coli	ATP standard solution	48
E. Nitric acid (0.1 N)	E. coli	ATP standard solution	50
F. Sulfuric acid (0.2 N)	E. coli	ATP standard solution	94
(0.6 N)	E. coli	ATP standard solution	99
(0.2 N)	Soil	ATP standard solution	<2
(0.6 N)	Soil	ATP standard solution	22
(1.5 N)	Soil	ATP standard solution	63
(1.5 N)	Solution	^{3}H-ATP	55
(1.5 N)	Soil	^{3}H-ATP	12

formate for Dowex), and these inhibit luciferase. Three washes with 50% ammoniacal ethanol releases 61% of the ATP from charcoal, and since ethanol is volatile its inhibitory effects can be minimized by evaporation. Some commercial luciferase preparations appear less susceptible to ethanol inhibition than others. This is being investigated further.

Column purifications are laborious and time consuming; comparisons were made of batchwise charcoal cleanup versus column purification using (^{3}H)-ATP in the presence and absence of soil. About 97-99% of ATP is adsorbed by charcoal whether in columns or batch. The presence of inert cellulose does not adversely affect the adsorption or release of ATP and it facilitates the packing of columns. Some 73-74% of (^{3}H)- ATP was either lost in the extraction procedure or adsorbed by the soil. Less ATP is adsorbed by the charcoal in presence of soil (75-80%) than without (95%). The recovery of ATP was the same (46-55%) from either columns or batchwise treatments. Since the columns are more time consuming and not significantly better, the batchwise treatment is the method of choice.

6. Applications

ATP has successfully been measured in E. coli, B. subtilis cultures, in plant tissues, in spring water samples, in microcosm samples and soil samples (both locally obtained ones and core samples and column samples from Dr. John Wilson of RSKERL, Ada, OK). The amount of ATP measured and the extraction method used are summarized in Table 16.

B. Lipopolysaccharide Extraction

1. Soil and Core Samples

Soil was extracted with 44% phenol at 68° C in a shaking water bath for 10 min. The pooled aqueous layers were extracted 2 X with ether to remove residual phenol. Then the LPS was determined as described in Section 5. Certain soil samples were spiked with known amounts of LPS to permit an internal standard measurement that was used for correction.

In a core sample collected by Dr. John Wilson we found 1.85 ng/g of material; in a Webster garden soil sample 85 ng/g of soil was found.

2. Water Samples

When an extensive dilution series was made on eastern Oklahoma spring water samples, we found that the previously determined values were underestimated. This occured because the signal due to gel formation determined photometrically saturates at a low absorbance value, unlike that observed in other spectrophotometric determinations.

TABLE 16. APPLICATION OF VARIOUS EXTRACTION PROCEDURES FOR ATP

Extraction Method	Sample	ATP Measured *	Recovery (%)
Boiling Tricine	*E. coli*	5.7 fg/cell	84
	B. subtilis	4.6 fg/cell	55
DMSO	*E. coli*	0.74 ng ATP/ 10^6 cells	39
	Soil sample	3.8 ng/g	
H_2SO_4	*E. coli*	1.6 fg/cell	74
	Soil sample	12 ng/g	59
	Wilson core	8 ng/g	57
	Wilson column	2 ng/g	63

*Corrected for inhibition and dilution.

What is required for accurate estimation of LPS is an extensive dilution series. When this was done we found 3.6 ng/ml in Grotto-Eureka water and 10 μg/ml in Park Spring water. For these environmental samples the less sensitive lots of LAL reagents may be useful.

C. Other Procedures -- Application to Spring Water

Spring water was collected in 8 locations in eastern Oklahoma and western Arkansas in sterile and pyrogen-free ampules. The samples were stored in a cold room (4° C) until assayed. Samples (1.0 and 0.1 ml) of the various spring waters were plated on nutrient agar for plate counts.

Sample preparation for colorimetric determinations consisted of adding 10 ml of 10% trichloroacetic acid and 5 mg of Hyflo-filter aid to 10 ml of water. The tubes were heated 15 min at 90° C and centrifuged, and the precipitate washed with 5% trichloroacetic acid. Centrifugation was repeated and the combined solutions were used for nucleic acid determinations (DNA with diphenylamine and RNA with orcinol). The protein in the precipitate was determined by the bromosulfalein method.

The amounts of several biochemicals found in spring water from several locations are shown in Table 17. Plating on nutrient agar showed a bacterial content of 2-473/ml. Since plating conditions could not be selected that would support the growth of all types of organisms, the plate count does not reflect the true bacterial count. There was no detectable DNA, RNA, or protein; the amounts were <1, <1, and <2.6 μg/ml, respectively (that indicates the lowest amount of the substances that could be detected). A fairly constant amount of lipopolysaccharide was found, but these samples were probably not diluted sufficiently; preliminary experiments suggest that this substance remains stable in the environment for a considerable time. ATP (determined by the firefly luciferase procedure) varied from 7 to 58 pg/ml, and heme (determined using luminol) from less than 2 to 200 pg/ml. Samples were measured for heme both before and after treatment with 0.5% hydrogen peroxide so that the response due to intact cells could be determined. Treatment of samples with 0.5% hydrogen peroxide destroys extracellular porphyrins and oxidizes reduced metal ions that may give a response but does not damage viable or intact cells (34).

Three conclusions can be drawn from the heme iron data. First, environmental samples contain measurable amounts of iron porphyrins. Second, hydrogen peroxide treatment substantially reduces the luminescence response of the samples. Last, there is no clear correlation between cell numbers measured by nutrient agar plate counts and amounts of iron porphyrins present in the samples. Each sample of spring water stimulated bacterial bioluminescence in the Microtox assay (data not shown).

There was no high correlation between any two of the components measured. This lack of correlation suggests that

TABLE 17. LEVELS OF SELECTED BIOCHEMICAL INDICATORS IN SPRING WATER SAMPLES

Source	Components (amount / ml)						
	Bacterial Count	DNA µg	RNA µg	Protein µg	LPS fg	ATP pg	Heme pg
Whaley's	59	<1	<1	<2.5	11	58	9
Behind Speedy's	29	<1	<1	<2.5	9.9	30	20
Billy Brown's	219	<1	<1	<2.5	10.5	7	14
Grotto-Eureka	4	<1	<1	<2.5	10.5	16	17
Charlie Tanihill's	2	<1	<1	<2.5	9.8	34	200
Park Spring	5	<1	<1	<2.5	9.8	22	25
Harding-Eureka	114	<1	<1	<2.5	10.5	33	2
Murrell Home	473	<1	<1	<2.5	9.8	45	2

additional parameters will be required to adequately characterize these spring water samples. Further data must be obtained before standards may be set.

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